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13. ABSTRACT *(Maximum 200 Words)*

Our research focussed on investigating acentric, autonomously replicating DNA containing amplified oncogenes (double minute chromosomes, DMs) as such structures occur in a significant fraction of human cancers. We proposed to develop methods to rapidly isolate and genotype DMs, to identify drugs to eliminate DMs, and to identify the mechanism(s) by which they are eliminated. We developed strategies to specifically tag DMs in living cells to enable analysis of their behavior during the cell cycle to enable us to ascertain differences between acentric structures and normal chromosomes to aid in the development of DM elimination reagents. We also devised and implemented novel cell labeling strategies to develop tumor models to determine how DM containing cells contribute to tumorigenesis, and to ascertain whether agents that effect DM elimination in vitro reduces tumor cell viability in vivo. Powerful new strategies and molecular tools were made available to the research community to enable sophisticated analyses of normal and abnormal chromosomes in cancer cells, and to allow studies of the relationship between genotype, phenotype and drug sensitivity in vivo.

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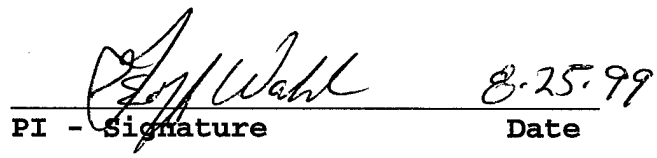

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2. INTRODUCTION

The overarching goal of this proposal has been to explore the feasibility of using a common type of genetic abnormality found in breast and other cancer cells as a target for chemotherapy. This type of genetic abnormality comprises acentric, autonomously replicating chromatin bodies called double minute chromosomes (DMs) (1). DMs encode oncogenes whose overexpression is important for the growth or survival of the cancer cells (1). As DMs lack centromeres, it is reasonable to infer that they must be subjected to continuous selection for their maintenance in the cell. DMs are known to arise from chromosome breakage, and cell cycle checkpoints and repair mechanisms present in normal cells are known to prevent their formation in normal cells (2,3,4). By contrast, loss of such control mechanisms occurs frequently during multistep cancer progression, and allows DM formation to occur at high frequency in the majority of cancer cell lines. Importantly, some of the most widely used antineoplastic agents can actually induce the chromosome breaks that lead to the formation of DMs and other aberrant chromosomes containing amplified genes (e.g., see (5). The activation of diverse oncogenes that occurs during tumor progression can also induce genetic instability (6,7,8). It is possible that certain types of anticancer treatments may collaborate with oncogene changes to enhance genetic instability and accelerate tumor progression. Thus, tumor cells constitute "moving targets" for therapy due to their genetic plasticity, and the ability of external stresses to induce genomic change. These factors justify the development of new treatment strategies that target essential genetic alterations that are peculiar to cancer cells to achieve greater selectivity and effectiveness, and to thereby reduce unwanted toxicity towards normal cells. DMs, and the oncogenes they encode, are the genetic alterations targeted by our studies supported by this grant.

3. SPECIFIC AIMS AND SUMMARY OF PROGRESS

Due to reductions in the time requested for funding, as well as in the amount of funds awarded, a modified Statement of Work with five Specific Aims was submitted in December, 1995. Progress towards the accomplishment of each is summarized below. Brief summaries are presented in cases where publications resulted from work performed. In addition, the data obtained suggested new research directions. Studies relevant to these new directions are also described below.

Specific Aim 1. Analysis of different types of agents that induce DM loss from human tumor cells

Previous studies showed that low concentrations of hydroxyurea (HU) induced the loss of amplified genes that localize to DMs, but not those integrated within chromosomes (9,10). Our previous studies indicated that DM loss was achieved through the formation of micronuclei (9,11,12). However, as HU is not a particularly effective anti-neoplastic agent, a primary goal was to ascertain whether other drugs would work as well or better. A related goal was to investigate the mechanism of HU-induced DM removal to provide a basis for the development of new drugs based on such mechanistic data.

The results of studies to address these questions have been published and will consequently be summarized briefly. A thorough description is given in the attached

reprint (12). We confirmed our earlier finding that DMs are selectively captured within micronuclei. Using micronucleation as a convenient assay, we found that many inhibitors of DNA replication effectively induced micronucleation, and that the micronuclei preferentially entrapped DMs. An important an unexpected observation was that such drugs did not induce micronuclei in normal fibroblasts containing a functional p53 pathway. However, abrogation of this pathway through expression of an oncogenic papillomavirus E6 protein, which targets p53 for degradation, increased the baseline micronucleation frequency and allowed the replication inhibitors to induce micronuclei to the same extent as found in cancer cell lines.

We next explored when in the cell cycle the micronuclei were generated. Cell cycle and microscopic analyses revealed that many micronuclei appeared to be generated during S-phase, not just after mitosis as had been thought previously (13,14,15). We observed buds that appeared to originate from the nuclear membrane, and that contained numerous DMs, but not centromeric fragments. Agents that work by diverse mechanisms to interfere with DNA replication were observed to induce DM loss and to increase the frequency of micronuclei and S-phase nuclear buds. We are still trying to understand the precise molecular mechanism by which replication inhibitors increase micronucleation.

These data led us to propose that there are at least two mechanisms for micronucleation. One involves the classical mechanism of capture of DMs within newly forming nuclear membranes as the DMs lag behind chromosomes at mitosis. The other occurs after mitosis, and appears to involve a novel budding process that can be induced by a variety of agents that interfere with DNA replication.

We have continued to develop more sophisticated methods to track DMs in living cells to enable us to explore the mechanism of micronucleation in greater detail. The long term goal of this project is to learn enough about the behavior of acentric elements to enable us to design better treatment strategies to eradicate them. To this end, we developed a novel transfection/recombination approach that has enabled us to target cloned DNA sequences into DMs at high efficiency. This procedure was described in detail in the last progress report, and is now reported in the appended draft of a manuscript to be submitted for publication soon (see Kanda and Wahl, 1999). Briefly, we adapted a procedure developed by Belmont and colleagues to identify specific chromosomal sequences in living cells through the interaction of tandemly arrayed lac operator (lacO) sequences with GFP-tagged lac repressor (lac R-GFP; (16). We found that vectors encoding the Epstein-Barr virus EBNA-1 protein, and containing the EBV replication origin, integrate at high frequency into DMs. A tandem array of 256 lac-operator sequences was cloned into this vector, and after transfection into COLO320DM cells, clones were selected that contain the EBV-lacO vector integrated into the DMs in this cell line. Subsequently, a vector encoding lac R-GFP was made and transfected into the cell line with lacO-tagged DMs to enable direct visualization of DMs in living cells. Brilliant dots were observed corresponding to the binding of the lac-repressor GFP to the DMs.

We are now using this system to analyze DM behavior in vitro and in vivo in tumors generated from this cell line. The preliminary data show that DMs occupy a peripheral nuclear location in prophase. As cells go through mitosis, the DMs relocalize, and occupy more terminal chromosomal positions, though not directly at telomeres. This causes many DMs to reside near the metaphase plate at mitosis. The DMs appear to be

tethered to distal chromosomal regions at this time, and they are also still double, or non-disjoined. Nondisjunction occurs as the chromosomes decondense and the cells enter G1. At some point in early-mid G1, the DMs redistribute so that they now appear to be randomly dispersed in the interphase nucleus, and they disjoin and are no longer "double". Occasionally, the DMs are freed from their chromosomal tether, and such free DMs can initiate micronucleus formation. The micronuclei appear to affix to the nuclear membrane, and this may explain why we saw them as "buds" in our previous analysis, which was done at much lower resolution. These data support the idea that DMs are able to track along chromosome arms by an as yet unknown mechanism, and that tracking and association are both regulated processes. Interestingly, we observed the same behavior for much smaller episomes containing only the EBV origin, lacO repeats, and encoding the EBNA-1 protein. We found that EBNA-1 protein is required for these small plasmids to tether to mammalian chromosomes, raising the possibility that DMs attach to chromosomes using a related mechanism involving protein-DNA interactions. We are currently exploring this possibility, and trying to identify the proteins that may be involved. Should this hypothesis be validated, we will try to interfere with the tethering mechanism to assess whether this increases micronucleation frequency.

The ability to observe DMs in living cells is now giving us the opportunity to observe the process of micronucleation in real time, and to investigate in greater detail how replication inhibitors enhance this process. We have observed DM bridges between mitotic figures. These bridges can be severed during cytokinesis. We are currently testing the hypothesis that breakage of such bridges produces damaged DNA which somehow attracts nuclear membrane components to seed the formation of micronuclei. We further hypothesize that replication inhibitors may increase the probability of breakage, or perhaps diminish repair to increase micronucleation frequency.

Specific Aim 2: Generation of animal models useful for studying DM behavior and the effects of DM elimination agents.

We have generated tumors in mice using COLO320DM and COLO32OHSR cells, and we found that the explanted tumors have a high frequency of micronuclei. We next wanted to produce tumors in which cells with DMs were labeled with one color, and cells with chromosomally amplified sequences (i.e., COLO320HSR) were labeled in another color. The purpose of this experiment was to develop a system useful for assessing whether cells with DMs exhibit different behaviors from those with HSRs in vivo. For example, the unequal segregation of DMs might enable such cells to occupy microenvironments different from those containing stably amplified genes that localize within chromosomes. This system would also enable us to ascertain whether the treatment strategies we develop selectively eliminate DM containing cells.

We have now generated tumors using various spectral variants of H2B-fluorescent protein fusion genes. We previously showed that H2B-GFP provides an extremely effective tool for labeling all nuclear DNA within a cell, and is sufficiently sensitive to enable detection of DMs (17). We made a variant of H2B in which the cyan fluorescent protein is employed (H2B-CFP), and another in which a yellow fluorescent protein (H2B-YFP) is used. H2B-CFP and H2B-YFP genes were cloned into retroviruses and stable DM and HSR cell lines were generated. These cells were then injected into nude mice and tumors produced. Figure 1 shows a thin frozen section depicting cells of both genotypes that are readily distinguished from each other. This experiment

documents that the fluorescent fusion gene is maintained stably and is expressed in these cells in vivo during the process of tumor cell growth. We have observed subregions of the tumors in which the DM cells predominate, and others in which HSR cells predominate. We are currently exploring whether these regions correlate with vascularization, oxygenation, etc. We have also generated tumors from cell lines in which the DMs are specifically labeled using the lacO-lacR-GFP strategy described above (Figure 2). This system should enable us to analyze the behavior of DMs in vivo, to assess the effectiveness of DM elimination agents in vivo, and to ascertain whether particular microenvironments select for an increase in DM content per cell.

We have extended the DM labeling strategy to other cell lines including SW527 (presumptive breast cancer), SW-613 (colon cancer) and CRL-2270 (neuroblastoma). The generality of the approach suggests that it may be feasible to attempt to label DMs in primary biopsy samples. The reason for this goal is to ascertain the generality of the findings with COLO320DM cells, and to overcome one of the severe limitations we have found in trying to analyze breast cancer cell lines. That is, over the years in which this grant was funded, we tried with minimal success to obtain breast cancer cell lines containing DMs. We found that the cell lines had either lost DMs, or that they were not breast cancer cells to begin with. The problem of DM loss during cell culture is generally observed, emphasizing the need to take cells directly from patients and establish them in an animal model, preferably using orthotopic transfer to establish growth in regions resembling those found in the human (18). We are now developing the collaborations to assess the feasibility of reaching this goal.

Specific Aim 3. Expand the types of tumors from which DMs can be isolated, and determine whether new genes are amplified

This is an ongoing set of experiments. We have obtained prostate, ovary, and neuroblastoma cells, and are analyzing them now for micronucleation. We are also attempting to use the lacO labeling strategy described above so that we can follow the behavior of the DMs in cell lines of diverse origins. We also plan to establish tumors in vivo from these cell lines, and to assess whether the DMs are maintained during in vivo passage. If this is observed, it would provide additional justification for attempting to establish nude mouse tumor models directly from patient biopsy material.

Specific Aim 4. Determine the consequences of DM elimination

We have not pursued this aim due to the inability to obtain breast cancer cell lines with DMs, and because of the additional time we spent developing the DM targeting technology described above. However, another group used the HU strategy to remove DMs from glioblastoma cells containing amplified epidermal growth factor receptors (19,20). This resulted in reduced growth, and induction of apoptosis in a subset of cells, as we observed in COLO320DM cells. This result suggests that induction of death may be a general consequence of purging cells of amplified genes. We will extend these studies to each cell line we obtain that contains DMs to assess the extent to which these observations can be generalized.

Specific Aim 5. Determining whether micronuclei serve as transfer vehicles for exchanging genetic information

The development of techniques to tag DMs with foreign DNA has enabled us to begin to assess whether micronuclei can facilitate the transfer of DM DNA to recipient tumor cells, and in this way accelerate chromosomal evolution. We made a derivative cell line

of COLO320DM, called S-12, in which DMs were tagged with the EBV vectors containing a GFP gene and a blasticidin selection marker. A two color FISH experiment confirmed the successful integration of the EBV vectors into DMs in S-12 cells. The tagged DMs in S-12 cells provide an optimal system for testing whether micronuclei can serve as transfer vehicles for DM-encoded information since the tagged S-12 DMs contain marker genes that can be tracked by either drug resistance or GFP expression in recipient cells. Micronuclei were purified from hydroxyurea-treated (100 μM, 3 days) S-12 cells as previously described (11). Purified micronuclei were overlayed onto WS1 normal human fibroblast (recipient cells) and they were processed for PEG-mediated cell fusion. The treated WS1 cells were expanded and selected with blasticidin. We expected that the successful transfer of DMs into WS1 cells would generate drug resistant colonies expressing GFP protein. However, no drug resistant colonies were obtained after drug selection. The failure of this experiment could be due degraded DNA in the micronuclei (see below) or an inappropriate cell fusion protocol. Another interesting possibility is that the C-Myc encoded by the transferred DMs could have induced cell cycle arrest in the normal recipient cells. Further experiments will be done using p53-deficinet recipient cells that should be immune to Myc-induced senescence or apoptosis.

The negative results obtained above led us to question whether the DNA in the micronuclei was intact. We have begun to investigate this problem by determining whether the micronuclei contain degraded DNA using the terminal transferase nick-end labeling procedure (TUNEL). Interestingly, under conditions where the nuclei are intact, we have detected a significant fraction of micronuclei that are TUNEL positive, indicating that the DNA they contain is significantly degraded (Figure 3). We next assessed whether there is any indication that these micronuclei are undergoing apoptosis. Indeed, we have found that the majority of TUNEL positive micronuclei are lamin negative (Figure 3). As nuclear lamins are substrates for the caspases that initiate apoptosis (21,22,23), the data suggest that there is a class of micronuclei that undergoes apoptosis under conditions where the main nucleus has its lamina intact and shows no chromatin condensation or other indications of apoptosis. These preliminary data suggest a new mechanism for localized degradation of nuclear DNA in the cytoplasm that is contained in a defective nuclear membrane. This finding may explain how DM DNA is lost from cells subsequent to micronucleation. Current experiments are designed to reveal how these micronuclei are targeted for localized apoptosis, and whether HU and other drug treatments increase the frequency of this process. We will also assess whether it requires the same proteins that participate in classical apoptosis, or whether new proteins are involved. A manuscript describing these data will be prepared upon completion of these experiments.

4. KEY RESEARCH ACCOMPLISHMENTS

- Development of a facile method for DM purification via isolation of micronuclei
- Demonstration of a new mechanism of micronucleation involving DM dissociation from chromosomes, and incorporation into micronuclei in interphase
- Demonstration that many classes of replication inhibitors increase micronucleation frequency, and that this process only occurs in cells that are devoid of p53 function

- Development of a sensitive method for labeling cells in different colors using H2B tagged with different colors of the jellyfish fluorescent protein; this method also provides a valuable and sensitive means of analyzing chromosome behavior and segregation in living cells
- Production of tumor cells of different genotypes that can be distinguished by their color and are useful for studying tumorigenesis and effects of drug treatment in vivo
- Development of tumors in mice in which it is possible to distinguish DM and HSR containing cells
- Development of a novel method for direct fluorescent tagging of DMs
- First analysis of DM behavior in living cells growing in tissue culture and in tumors in mice; the in vitro studies provide the first direct evidence that DMs relocalize in the nucleus during the cell cycle, and that they attach to chromosomes and track along chromatids in a fashion similar to small DNA virus replicons

5. REPORTABLE OUTCOMES

Manuscripts:

•Shimizu, N., Kanda, T., and Wahl, G.M. (1996) Selective capture of acentric fragments by micronuclei provides a rapid method for purifying extrachromosomally amplified DNA. Nature Genetics. 12: 65-71.

•Shimizu, N., Itoh, N., Utiyama, H., and Wahl, G.M. (1998) Selective entrapment of extrachromosomally amplified DNA by nuclear budding and micronucleation during S-phase. J Cell Biol. 140 (6): 1307-1320.

•Kanda T., Sullivan, K.F., and Wahl G.M. (1998) Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living cells. Current Biology 8: 377-385.

•Kanda, T., and Wahl, G.M. (1999) Visualization of the mitotic behavior of Epstein-Barr virus vectors and double minute chromosomes in human cells (draft of manuscript enclosed).

Inventions and Patents:

Geoffrey M. Wahl, Teru Kanda, Noriaki Shimizu
Method for Isolation of Extrachromosomal Amplified Genes
Serial Number 452,275, Filed May 26, 1995

Geoffrey M. Wahl, Teru Kanda, Noriaki Shimizu
Method for Isolation of Extrachromosomal Amplified Genes
Serial Number 08/704,391, Filed August 16, 1996

Degrees: none

Cellular and molecular reagents:

H2B-GFP, H2B-CFP, H2B-YFP vectors and retroviruses
Cell lines expressing these fluorescent proteins (HeLa, COLO320DM, HSR)
Targeting vector for DMs encoding EBNA-1, and containing lacO tandem array
Expression vector encoding lac repressor GFP

Funding applied for and received:
California Cancer Research Program
-- (Wahl)
Novel Treatment Targeting Cancer Cell Genetic Instability
01/01/99-12/31/04
$175,474 (annual direct cost)

Bosch Foundation
--(Wahl)
Gene Amplification in Double Minute Chromosomes (DMs): A Target for Treatment of Solid Tumors
07/01/99-07/01/01
$93,200 (total direct cost [2 yrs])

6. CONCLUSIONS

The studies supported by this grant have given immense insight into the biology of double minute chromosomes (DMs), one of the most frequently observed genetic abnormalities in human cancers. Techniques were developed to allow the rapid and efficient isolation of DMs from many types of tumor cell lines, and from direct tumor explants without the need for cell culture. Efficient PCR-based methods were developed to enable the localization of the chromosomal sequences from which the DMs were formed. This approach could find significant applications in tumor grading, prognosis, and therapeutic decision making. The intracellular behavior of DMs during the cell cycle was analyzed using sophisticated microscopic strategies, combined with newly developed techniques that enable the tracking of DMs within living cells in real time. We found that DMs have a propensity to associate with chromosomes, and that the association seems to be regulated during the cell cycle. DMs localized to the periphery of the nucleus in prophase, and then went to near terminal chromosomal regions during mitosis. The DMs seemed to relocalize to random positions during G1. Importantly, we found that a small, circular DNA virus replicon exhibited similar behavior, and that the association with chromosomes was mediated by a viral protein involved in DNA replication binding to its cognate viral replication origin. These data suggest that DMs may also tether to chromosomes through protein-DNA interactions. We also found that DMs can be lost from cells by incorporation into micronuclei, and that the frequency of micronucleation and DM loss can be increased by many agents that interfere with DNA replication. This process is dependent on cells having defective p53 mediated cell cycle control. As many of the drugs we found to effect DM loss are in the categories currently used in chemotherapy, we expect that they would have collateral, unwanted side effects on normal cells. It is of great interest, therefore, that the chromosome tethering behavior displayed by DMs appears to be unique for acentric elements. This may enable development of DM-specific elimination reagents that could have greater selectivity for cancer cells than standard chemotherapeutic agents that work on targets also found in normal cells. Identification of such agents is a continuing goal of the research stimulated by this award. Finally, the sophisticated molecular strategies we developed to study DMs in living cells have also made available cancer cell lines labeled with different fluorescent

tags, which can be customized for the specific genotype of the cell to be analyzed. We developed tumor models using these labeled cancer cells, and we are beginning to detect differences in the behavior of the cells in vivo. These studies offer great promise for elucidating how particular genetic changes contribute to tumor establishment or metastasis, to targeting of tumor cells to particular microenvironments, and to identifying which drugs work best against specific genetic defects. This opens the way for more rational therapeutic approaches in the future. And for designing therapies targeted against cancer-specific genetic defects.

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8. APPENDICES:

A. Figure legends and Figures - see attached

B. Reprints and manuscripts – see attached

C. CV – see attached

D. Patent Applications – see attached

Fig. 1

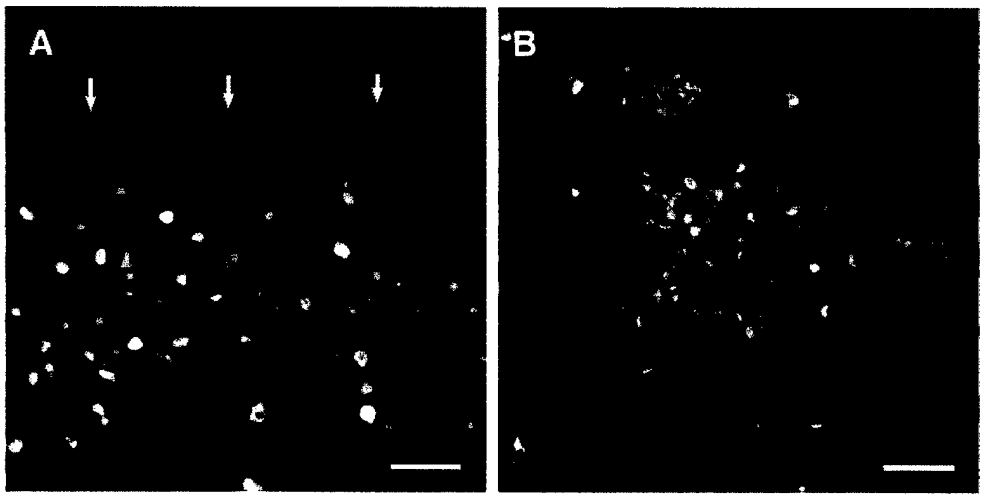

Figure 1: Cells containing DMs or HSRs can be tracked after generating tumors in nude mice

COLO320DM cells expressing H2B-CFP and COLO320HSR cells expressing H2B-YFP were made by retroviral infection in vitro. Mixed population of differentially labeled DM cells and HSR cells ($1x10^7$ cells each) were injected into 6 week-old BALB/c nu/nu female mice. Tumors (approximately 1.5 cm) were excised and processed for histological examination in frozen sections. Samples were observed using a fluorescent microscopy (20x objective) equipped with appropriate filter sets for detecting CFP and YFP. (A) HSR cells (yellow) were more predominant than DM cells (blue) at the periphery of the tumors. The surface of the tumor is indicated by arrows. The scale bar is 50 μm. (B) DM cells (blue) were found to be more predominant than HSR cells (yellow) in a certain area inside the tumor.

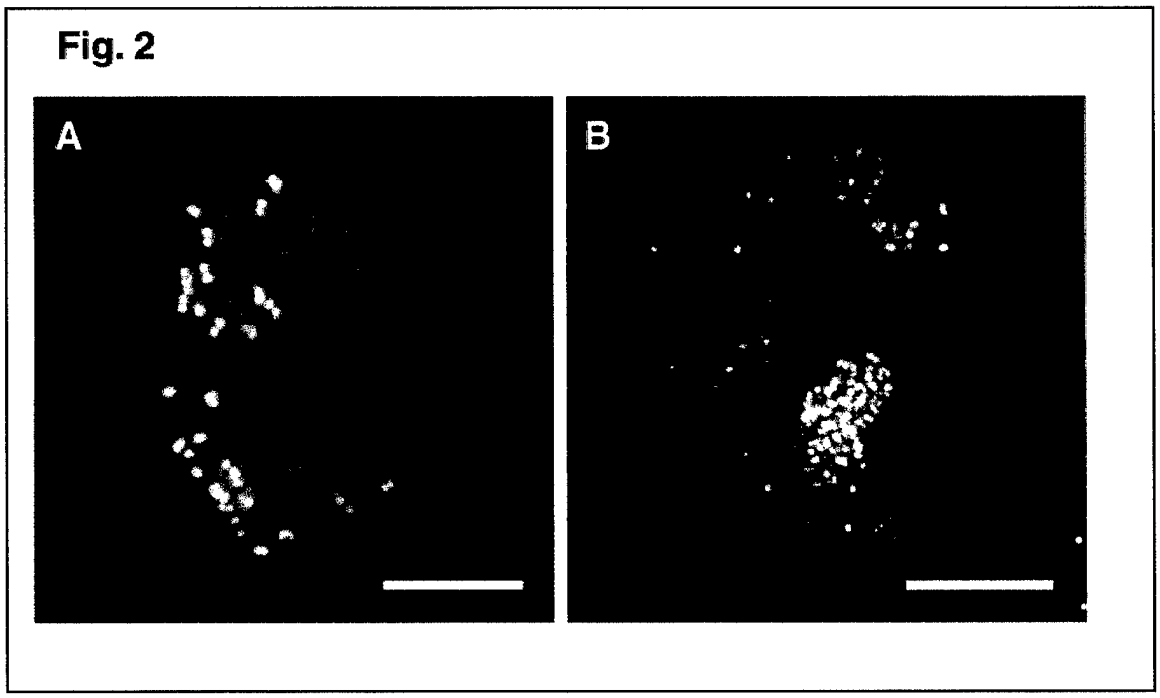

Fig.ure 2: DMs can be readily identified in tumors growing in nude mice
COLO320 DM cells, in which DMs were labeled with integrated lac operator repeats, were infected with two different retroviruses, one expressing H2B-CFP and the other expressing lac repressor-YFP, simultaneously. The infected cells ($2x10^7$ cells) were injected into 6 week-old BALB/c nu/nu female mice. Frozen sections of the tumors were observed using fluorescent microscopy (60x objective for A and 100x for B). (A) Paired dots of DMs (lac repressor-YFP labeled, yellow) were found to be at the periphery of the prophase chromosomes (H2B-CFP labeled, blue). The scale bar is 5 μm. (B) A large cluster of DMs (yellow) was found to tether to a group of segregating chromosomes (blue) in an anaphase cell. These rsults confirm in vivo what we previously observed in cells growing in cell culture.

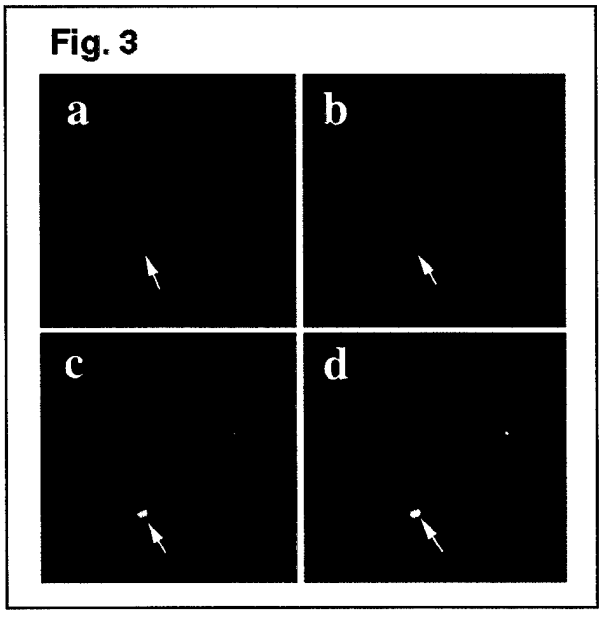

Figure 3: Selective DNA degradation in cytoplasmic micronuclei
COLO320DM cells were ficed and then stained with DAPI (DNA) (a), anti-lamin antibodies (b), and assayed by TUNEL (c) for degraded DNA. Note that the degraded micronuclear DNA stains positively by TUNEL and negatively for the lamins (b and c). The nucleus, surrounded by lamins (b) stains negatively by TUNEL (c) (d, merged image). Arrows indicate the micronucleus.

Visualization of the mitotic behavior of Epstein-Barr virus vectors and double minute chromosomes in human cells

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Abbreviations

DMs, double minute chromosomes; EBV, Epstein-Barr virus; GFP, green fluorescent protein; DAPI, 4',6'-diamidino-2-phenylindole; VSV-G, vesicular stomatitis virus G glycoprotein; FISH, fluorescence in situ hybridization;

Abstract

Double minute chromosomes (DMs) are paired acentric chromatin bodies that contain amplified oncogenes and they are frequently observed in human cancer cells. DMs accumulate to a high copy number because of their autonomous replication and subsequent uneven distribution to daughter cells. We previously found that DMs associated with mitotic chromosomes in living cancer cells. Recent data indicate that similar associations occur with extrachromosomally replicating viral vectors. We examined the behavior of Epstein-Barr virus (EBV) vectors, containing the viral DNA replication origin (oriP) and the EBNA-1 gene, by adding 256 direct repeats of the lac operator to the vector (EBV-lacO vector). The vector can be visualized in cells expressing a fusion protein consisting of the green fluorescent protein (GFP) and the lac repressor (lacR-GFP). We found that the vector associated with mitotic chromosomes, and the association was disrupted by deleting EBNA-1 gene from the vector. Surprisingly, transfection of the EBV-lacO vector into DM-harboring cells revealed their preferential recombination with DMs, but not with normal chromosomes. DMs containing the integrated lac operator repeats were readily visualized by lacR-GFP staining. We used this system in combination with immunological detection of centromeric regions and mitotic spindles to further examine the mitotic behavior of DMs. In prophase cells, DMs aggregated and associated with the distal chromosomal arms at the nuclear periphery, while centromeric regions were pulled inward by the attached mitotic spindles. Sister minute chromosomes did not dissociate while normal sister chromatids were pulled apart by the attached spindle microtubules at metaphase-anaphase transition. DMs linked to distal chromosomal arms were efficiently incorporated into daughter nuclei. For real time analyses, we labeled entire chromosomes with histone H2B-CFP (cyan GFP) and specifically labeled EBV-integrated DMs with lac repressor YFP (yellow GFP). The result further confirmed the peripheral relocation of DMs during prophase and their chromosome tethering throughout mitosis. Some sister minutes clearly showed nondisjunction until early G1 phase, and they subsequently dispersed throughout nuclei. Our observation that both viral and cellular extrachromosomally

replicating molecules tether to mitotic chromosomes implies that chromosome tethering is a common mechanism for their efficient nuclear retention during mitosis.

Introduction

Replication and subsequent faithful segregation of the replicated chromosomes are crucial for the proper transmission of the cellular genome to daughter cells. In higher eukaryotes, mitotic spindle attachment to kinetochores, formed at centromeres of chromosomes, assures even distribution of sister chromatids. It has been shown that multiple checkpoint mechanisms, such as DNA repair checkpoint and mitotic spindle checkpoint, exist throughout the cell cycle to keep the integrity of the genome. However, in cancer cells, disruption of such checkpoint mechanism sometimes allows abnormal chromatin bodies to be extrachromosomally transmitted to daughter cells. Such extrachromosomally maintained chromatin bodies are called double minute chromosomes (DMs). DMs are cancer-specific genomic anomalies and known to harbor amplified oncogenes and drug resistance genes (Cowell, 1982). They are believed to evolve from submicroscopic autonomously-replicating circular precursors which are clipped out from their native chromosomal loci by improper repair of double strand DNA breaks (Wahl, 1989; Windle et al., 1991). In tumor biopsies, DM-mediated gene amplification is more prevalent than intrachromosomal gene amplification (homogeneously stained regions) (Benner et al., 1991).

DMs are usually 1-2 Mb in size and can be observed as paired dots in fixed chromosome spreads by light microscopy. Electron-microscopic observation has demonstrated that DMs are circular chromosomes lacking centromeres and telomeres (Barker and Stubblefield, 1979; Hamkalo et al., 1985; Jack et al., 1987; Rattner and Lin, 1984). DMs contain at least one functional replication origin and replicate once per cell cycle according to the cellular program (Carroll et al., 1991). Therefore, paired-dots of DMs observed in metaphase spreads are most likely replicated sister molecules. The copy number of DMs varies from cell to cell (zero to hundreds), suggesting that they are unevenly distributed among daughter cells during mitosis due to their lack of centromeres. Uneven distribution of DMs can have direct impact on their copy number change, and overexpressed gene products resulting from increased copies of DMs should contribute to growth advantages during tumor progression. Therefore, studying the mitotic behavior of DMs should give

us an important clue for understanding how cancer cells can increase malignant potential during tumor progression.

High level accumulation of DMs in tumor cells in vivo implies that DMs utilize a special mechanism to be efficiently transmitted to daugther cells even though they lack functional centromeres. Light and electron microscopic observation of fixed tumor cells demonstrated that DMs were frequently associated with mitotic chromosomes (Barker and Hsu, 1978; Hamkalo et al., 1985; Jack et al., 1987; Levan and Levan, 1978). This observation has been validated by our recent obervation of DMs in living cancer cells (Kanda et al., 1998). We fluorescently labeled entire chromosomes, including DMs, in vivo by expressing a fusion protein of human histone H2B and *Aequorea victoria* green fluorescent protein (H2B-GFP). DMs, which were identified as small fluorescent dots (up to one micron in diameter), frequently made clusters and tethered to segregating daughter chromosomes in anaphase cells. Time-lapse microscopy revealed that DMs were actually 'hitch-hiking' on segregating chromosomes from anaphase to telophase and efficiently incorporated into daughter nuclei (Kanda et al., 1998).

Latent infection of Epstein-Barr virus (EBV) is another example of extrachromosomally maintained DNA molecules in human cells. The EBV genome is a 170 kb double-stranded circular episome in its latent infection (Mecsas and Sugden, 1987). Plasmid vectors based on EBV, which consist of latent origin of replication (oriP) and the viral transactivator protein EBNA-1, are stably maintained extrachromosomally in human cells over long periods of time (Yates et al., 1985). OriP is composed of two clusters of EBNA-1 binding sites; the family of repeats (FR) and the dyad symmetry element (DS) (Reisman et al., 1985). Interestingly, incorporation of FR element into plasmids lowers the rate at which they are lost from cells expressing EBNA-1, although the plasmids are replication incompetent, demonstrating that FR and EBNA-1 cooperatively work for nuclear retention of the plasmids (Middleton and Sugden, 1994).

Accumulating evidence demonstrate that EBV vectors tether to mitotic chromosomes, and the tethering is mediated by EBNA-1 protein. Fluorescence-activated chromosomes sorting and fluorescence in situ hybridization (FISH) revealed that EBV vectors actually associated with mitotic

chromosomes (Harris et al., 1985; Simpson et al., 1996; Westphal et al., 1998). As EBNA-1 protein is known to localize on mitotic chromosomes (Grogan et al., 1983; Petti et al., 1990), it is likely that EBNA-1 can link oriP-containing vectors and mitotic chromosomes. Chromosome tethering should facilitate the efficient incorporation of the EBV vectors into daughter nuclei when nuclear membranes reform at the end of mitosis. Recently, such chromosome tethering has also been demonstrated for bovine papillomavirus (BPV) (Ilves et al., 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998), and for Kaposi's sarcoma associated herpesvirus (KSHV) (Ballestas et al., 1999). BPV and KSHV have their own cis-acting sequence and trans-acting viral proteins, E2 and LANA, respectively, which mediate their chromosome tethering. Therefore, chromosome tethering may be a common mechanism with which extrachromosomally replicating viruses are efficiently transmitted into daughter nuclei.

We found several common features between DMs and EBV vectors; they are both circular molecules, replicate only once per cell cycle (Yates and Guan, 1991), and they both tether to mitotic chromosomes. The similarity between the behavior of DMs and that of the EBV vector prompted us to test whether they behave similarly when they co-exist in the same cells. We employed previously described lac operator/lac repressor-GFP system (Robinett et al., 1996) to rapidly identify the localization of the EBV vector. This system allowed us to readily visualize the chromosome tethering of the EBV vectors, which have lac operator repeats, in the transfected cells. Transfection of the same vector into DM-harboring cells, followed by drug selection, revealed that the vector preferentially recombined with DMs but not with normal chromosomes. DMs with integrated EBV vectors were readily detected by lac repressor-GFP staining. We used this strategy, in combination with conventional immmunofluorescence analyses, to clarify the detailed mitotic behavior of DMs relative to those of centromeres and spindle microtubules. Moreover, dual-color GFP labeling enabled real-time observation of entire chromosomes and DMs throughout cell cycle. Frequent recombination between DMs and EBV vectors implies that cellular (DMs) and viral extrachromosomally replicating DNAs occupy the same intranuclear territory, which contributes to the high probability of their recombination.

Materials and Methods

Plasmid construction

EBV oriP and EBNA-1 coding sequences in EBV-lacO vector were derived from pCEP4 (Invitrogen). A fragment encoding hygromycin resistance gene, CMV promoter, and SV40 polyadenylation signal was first deleted from pCEP4 by SalI (blunted by Klenow enzyme)- NruI digestion followed by self-ligation to make pEP4, in which the SalI site recovered after self-ligation. A blasticidin resistance gene (Izumi et al., 1991), driven by the SRα promoter, was clipped out from pYN3215-bsr (kindly provided by Dr. Fumio Hanaoka, Osaka University) by NheI digestion and subcloned into XbaI site of pEP4 to make pEPB. Subsequently, an EcoRI-SalI fragment of pEPB, containing blasticidin resistance gene, oriP, and EBNA-1 gene, was subcloned into EcoRI-SalI digested pMBL19 to make pMBL19EBVbsr. pMBL19, which has a bacterial p15A ori, was chosen for its ability to subclone unstable inserts (Nakano et al., 1995). Lac operator repeats (256 direct repeats) were obtained by SalI-XhoI digestion of pSV2-dhfr 8.32 (Robinett et al., 1996). The repeats were subcloned into the SalI site of pMBL19EBVbsr to make EBV-lacO vector. This step was carried out using STBL2 competent cells (Life Technologies GIBCO BRL) grown at 30 °C (Belmont et al., 1999). The EBV-lacO vector was digested with MfeI-ClaI (blunted by Klenow enzyme) followed by self ligation to make the vector lacking EBNA1 gene.

A splicing retroviral vector expressing lac repressor-GFP (lacR-GFP) was constructed in order to take advantage of highly efficient translation. pCLMFGMCS (kindly provided by Dr. Nikunj Somia, Salk Institute) is identical to pMFG vector (Dranoff et al., 1993) except that it has CMV promoter instead of U3 region of 5' LTR and that it has multiple cloning site (NcoI-EcoRI-SalI-XhoI-NotI-BamHI). pCLMFGlacR-GFP was constructed by ligating (1) NcoI-BsrGI fragment of pEGFPN1 (Clontech), (2) BsrGI-DraI fragment of p3'SSdimerClonEFP (containing a gene encoding lac repressor-nuclear localization signal (Robinett et al., 1996), and (3) XhoI (blunted by Klenow)-NcoI digested pCLMFGMCS. For expressing lacR-YFP, pCLMFGlacRYFP was constructed in the same way using NcoI-BsrGI fragment of pEYFP (Clontech).

A histone H2B-CFP fusion gene was made by swapping AgeI-NotI fragment of H2B-GFPN1 (Kanda et al., 1998) (which has a Clontech backbone vector) with AgeI-NotI fragment of pECFP (Clontech). NotI (blunted by Klenow enzyme)-HindIII fragment containing the H2B-CFP gene was subcloned into ClaI (blunted by Klenow enzyme)-HindIII digested pCLNRX vector. (Naviaux et al., 1996).

Cell lines and transfection

A stable HeLa cell line expressing lacR-GFP protein was established by infecting lacR-GFP retrovirus and limited dilution, followed by single colony isolation. HeLa cells expressing lacR-GFP were transfected with the EBV-lacO vector using a calcium phosphate precipitation protocol.

COLO320DM cells harboring DMs containing an amplified c-myc gene were grown as described (Kanda et al., 1998). Exponentially growing cells ($1x10^7$) were transfected with 5 μg of the EBV-lacO vector using electroporation (BioRad), resuspended in 10 ml of culture medium, and plated into two 10 cm dishes (8 ml, 2 ml for each dish). Blasticidin (15 μg/ml) was added to the transfected cells 24 hrs after transfection, and cells were selected for 14 days. Drug resistant cells were further grown under reduced blasticidin concentration (5 μg/ml), and blasticidin resistant colonies were isolated and replated into 48 well dishes. Cells were expanded in media containing blasticidin (5 μg/ml), and fast growing 12 clones were selected and used for retroviral infection and further expansion. This strategy was repeated independently three times to confirm the reproducibility of the experimental data.

Production of VSV-G pseudotyped retrovirus was performed using four 10 cm plates of 293 gp/bsr cells (2 x 106 cells) as packaging cells (Miyoshi et al., 1997). For each dish, 15 μg of retroviral vector and 5 μg of pMD.G (the plasmid encoding the envelope protein VSV-G) were co-transfected using a calcium phosphate precipitation protocol. Fresh medium (9 ml per dish) was added 24h after transfection, and virus containing supernatants (9 ml x 4 plates = 36 ml) were collected 72h, 96h, and 120h after transfection. The virus containing supernatants were ultra-centrifuged (25000 rpm, 90 min, 4 degree) using a SW28 rotor, and pelleted virus was resuspended

in 400 µl PBS, and stored in aliquots at -80 degree until use. After concentration by ultracentrifugation, titers of 1 x 10^7 infectious units / ml on HeLa cells were usually obtained.

For virus infection, recipient cells were plated in either 12 well dishes (with 1 ml medium) or in 6 well dishes (with 2 ml medium). Concentrated viral supernatants (20 µl/ml medium) were added to recipient cells every 12 hrs in the presence of 8 µg/ml polybrene (Aldrich). After overnight incubation, medium was changed and the infected cells were expanded for further analyses. Double infection of H2B-CFP and lacR-YFP viruses was performed by simultaneously adding two kinds of concentrated virus solution to recipient cells.

Fluorescence in situ hybridization (FISH)

Cells were treated with colcemid (100 µg/ml) for 50 min, and chromosome spreads were prepared by conventional fixation. For dual color FISH, c-myc cosmid DNA was labeled with biotin, while lac operator repeat (SalI-XhoI fragment of pSV2-dhfr 8.32) (Robinett et al., 1996) was labeled with digoxigenin using random prime labeling. Denaturation, hybridization, and washing were performed as previously described (Shimizu et al., 1996). Signals were detected using FITC-avidin (10 µg/ml) and Rhodamine conjugated sheep anti digoxigenin antibody (4 µg/ml).

For fiber-FISH, chromatin fibers were prepared on slide glasses as previously described (Parra and Windle, 1993). Signals were detected using 3 sequential steps of signal amplification protocol as follows. (1) FITC-avidin (5 µg/ml) and anti digoxigenin monoclonal antibody (1 µg/ml), (2) biotinylated goat anti-avidin (1 µg/ml) and digoxigenin labeled sheep anti mouse IgG (2 µg/ml), (3) FITC-avidin (5 µg/ml) and Rhodamine conjugated sheep anti digoxigenin antibody (4 µg/ml).

Southern blot analyses

Genomic DNAs (10 µg), with and without appropriate amount of mixed EBV-lacO plasmid DNA, was digested with HindIII, which clips out 10 kb fragment of lac operator repeats from the EBV-lacO plasmid. Digested DNA samples were analyzed by Southern blotting using ^{32}P labeled lac operator repeat (SalI-XhoI fragment of pSV2-dhfr 8.32) (Robinett et al., 1996) as a probe.

Western blot analyses

Cells (1 x 10^7 cells in 10 cm dish) were lysed in 800 µl of buffer containing 3% SDS, 125 mM Tris (pH 6.7), 6% urea, and 10% glycerol with 100 µg/ml PMSF and 3 µg/ml aprotinin. Lysed cells were sonicated and centrifuged to remove debris. Aliquots of whole cell extracts were resolved by SDS-10% polyacrylamide gels. Western blotting was performed using EBV-immune human serum reactive to EBNA (1:200 dilution) (kindly provided by Dr. George Miller, Yale Univ.), which recognizes EBNA-1 protein, as a primary antibody, and peroxidase conjugated goat anti human IgG (1:2500 dilution, Boehringer Mannheim) as a secondary antibody. Signals were detected by enhanced luminol reagents (NEN Life Science Products) according to the manufacturer's instructions.

Immunofluorescence staining

Cells, harboring DMs labeled with lacR-GFP, were attached to slide glasses by cytospin (500 rpm, 1 min) and fixed with 3.7% formaldehyde for 10 min. Slides were washed with PBS three times, and treated with blocking buffer (2.5% BSA, 0.2M glycine, 0.1% TritonX-100) for 30 min.

Primary and secondary antibodies were diluted in the blocking buffer. Primary antibodies used for centromere, microtubules, and EBNA-1 staining were: human CREST autoantiserum hACA-M (1:2000 dilution) (Sullivan et al., 1994), monoclonal anti α-tubulin (1:2000 dilution, Sigma), and EBV-immune human serum (1:200), respectively. Following incubation for 60 min at room temperature, slide glasses were washed three times with PBS. Secondary antibodies were Cy5 conjugated goat anti human IgG (1:1000, for centromere and EBNA-1 staining) and rhodamine conjugated anti mouse IgG (1:1000 dilution, for microtubules staining). Following incubation for 60 min at room temperature, slide glasses were washed three times with PBS, and chromosomes were counterstained with DAPI (1 µg/ml). Fluorescence of lacR-GFP was completely preserved by this protocol.

Microscopy

All images appear in this article were collected using DeltaVision microscope system (Applied Precision Inc. Issaquah, Washington, USA) using either 63x/NA 1.4 or 100x/NA 1.35 oil immersion objectives (Olympus).

For fixed specimens (except for the images of Fig. 3), three-dimensional data sets were collected to visualize EBV vectors and DMs as they distributed in multiple focal planes. Optical sections were collected at 0.2-μm focal intervals; pixel size was 0.111 μm for 63x objective (Fig. 3, 4, 5, and 6A) and 0.0669 μm for 100x objective (Fig. 2). Out-of-focus contamination was removed from each optical section (called deconvolution processing) and two-dimensional images were created by projecting the three-dimensional data stacks using the software supplied with DeltaVision system.

For live observation, cells were grown on 40 mm cover slips, which were pretreated with fibronectin solution (25 μg/ml in PBS), and mounted with prewarmed medium (containing 20 mM HEPES pH 7.3) to a FCS2 chamber system (Bioptechs, Butler, PA). Special filter sets required for CFP and YFP detection (Ellenberg et al., 1999) were installed into the DeltaVision microscope system. Single slice images were collected every minute using either 63x (Fig. 6B) or 100x objective (Fig. 6C) equipped with an objective heater (Bioptechs). Pixel size was 0.222 μm (63x) or 0.1338 μm (100x) as binning factor 2 was used to minimize the total exposure time during observation.

Results

Chromosome tethering of the EBV vector

Although FISH protocols have been used to demonstrate that EBV vectors associated with mitotic chromosomes (Simpson et al., 1996; Westphal et al., 1998), they are laborious protocols and do not preserve fine chromosomal structures as they require DNA denaturation for hybridization. Therefore, we employed the lac operator/repressor recognition system (Robinett et al., 1996) so that we can quickly examine the localization of the EBV vectors under less harsh condition. We added 256 direct repeats of the lac operator (lacO) to the vector (EBV-lacO vector)

for rapid identification of its intracellular localization (**Fig. 1 left**). A retroviral vector expressing lac repressor protein fused to green fluorescent protein (lacR-GFP) was used for identifying the transfected EBV-lacO vectors as fluorescent dots (**Fig. 1 right**).

HeLa cells stably expressing lacR-GFP protein was established (**Fig. 2A**), and the cells were transfected with either the EBV-lacO vector or the vector lacking EBNA-1. Mitotic cells were collected 6 days after transfection and examined for the distribution of the intracellular fluorescent dots. We found that EBV-lacO vectors extensively associated with mitotic chromosomes (**Fig. 2 C, D, E**), supporting the previous FISH results (Simpson et al., 1996; Westphal et al., 1998). Fluorescent dots appeared to be distributed randomly on mitotic chromosomes, suggesting that the EBV-lacO vector did not bind specific chromosomal regions, such as centromeres and telomeres. On the other hand, when the vector lacking EBNA-1 gene was transfected, fluorescent dots were found to be scattered around the cytoplasm and did not show specific association with mitotic chromosomes (**Fig. 2F**). This observation provides the first direct evidence that EBNA-1 mediates the association between oriP plasmids and mitotic chromosomes.

Blasticidin selection of the transfected cells revealed that the transformation efficiency obtained by the EBV-lacO vector was approximately 3×10^{-2}, which was more than 1000 fold higher than the one obtained by the vector lacking EBNA-1 gene (approximately 2×10^{-5}). Microscopic observation revealed that fluorescent dots of EBV-lacO vectors still associated with mitotic chromosomes in the drug resistant cells (data not shown). Highly efficient transformation and mitotic chromosome association correspond well with the stable episomal maintenance of the EBV lac-O vector. The result demonstrates that the lac operator/lacR-GFP system can be applied to track the transfected extrachromosomal DNAs, and it validates the existing model that chromosome tethering of the EBV vectors is mediated by EBNA-1 protein.

DMs are preferred targets for EBV vector integration

We next examined the fate of the EBV-lacO vector after transfection into DM-harboring cells. The EBV-lacO vector was transfected into COLO320DM cells, which have DMs encompassing c-myc loci. Transfected cells were selected with blasticidin, and drug-resistant colonies were isolated

and expanded into 12 independent cell lines. When aliquots of these cells were infected with retrovirus expressing lacR-GFP, punctate staining in nuclei were observed in all clones (data not shown). We chose three clones with the brightest fluorescent dots, expecting that these clones maintain abundant EBV-lacO vectors. We prepared chromosome spreads, and examined the localization of lacO repeats and DMs using the lacO repeat DNA and a c-myc cosmid as probes. Representative dual-color FISH images of one of the established clones are shown in Fig.3. The result revealed that the signals of lacO repeats overlapped with many of the DMs (labeled with c-myc probe) **(Fig. 3 A)**. We examined 50 metaphase spreads of one of the established clones and counted the number of DMs with overlapping lacO signals. We found that 50% (25/50) of the examined metaphase spreads showed complete colocalizations, and 90% (45/50) of the metaphase spreads had at least one pair of DMs with overlapping lacO signals **(Fig. 3 B)**. On the average, each chromosome spread contained 18.3 (± 19.2) pairs of DMs, and 12.7 (± 13.2) of them had overlapped lacO signals. Interestingly, lacO signal was never found to overlap with intrachromosomal c-myc signals **(Fig. 3A)**, strongly suggesting that DMs are the preferred targets for the colocalization of the transfected EBV vectors. We did not see any evidence of extrachromosomal free EBV vectors existing in the established cell lines, although we cannot exclude the possibility that our FISH protocol was not sensitive enough to detect such molecules.

The colocalization of DMs and lacO repeats could be due to covalent integration of the EBV-lacO vectors into DMs or due to noncovalent association. In order to clarify the nature of the colocalization, we prepared stretched chromatin fibers (Parra and Windle, 1993) using one of the established cell lines, and they were analyzed by dual-color FISH (lacO repeats and a c-myc cosmid as probes). We observed arrays of the lacO signals on the same DNA fibers on which c-myc signals were detected **(Fig. 3C)**. The results demonstrate that multiple copies of the lacO repeats are covalently integrated into DMs. Southern blotting showed that approximately 130 copies of the of lacO repeats existed per cell, and several extra bands other than the expected-size band (10 kb) were noticed **(Fig. 3 D)**. Thus we saw an average 12.7 DMs with integrated lacO repeats, each DM with approximately 10 (=130/12.7) copies of lacO vectors. We speculate that DMs have recombined

with multiple EBV-lacO vectors and become larger extrachromosomal molecules during drug selection. This explains why the EBV-integrated DMs were sometimes found to be bigger than those of the parental COLO320DM cells. The complex signal patterns observed by fiber-FISH, together with the existence of the extra bands observed in Southern blotting, indicate that the integration events may be complicated.

When we transfected the vector lacking EBNA-1 gene into COLO320DM cells, very few drug resistant colonies arose by the same transfection and drug selection. Moreover, FISH analyses of drug resistant cells revealed that the transgenes randomly integrated into normal chromosomes as well as into DMs (data not shown). Therefore, using EBV vectors with EBNA-1 gene appeared to be critical for efficient targeted integration into DMs.

Specific labeling of DMs using lac repressor-GFP and their mitotic behavior

The targeted integration of the EBV vectors has enabled the rapid detection of DMs by in vivo expression of the lacR-GFP fusion protein, which binds with high affinity to the lacO repeat sequence carried by the vector. A retroviral vector expressing the lacR-GFP fusion protein was used to infect the cell lines with lacO repeats integrated into DMs. Approximately 80% of the infected cells were found to express lac-R-GFP protein in their nuclei. Punctate staining in nuclei were observed in approximately 30% of the GFP expressing cells, while rest 70% of the cells showed diffuse nuclear staining, presumably due to the excess level of lacR-GFP expression. Fluorescence intensities of the punctate staining were much stronger compared to those observed in HeLa cells transiently transfected with the same vector (Fig. 1), corresponding well with the estimation that each DM pairs had approximately 10 copies of the lac operator repeats on the average (as described above).

This DM labeling strategy was used in combination with immunological detection of centromeres and mitotic spindles to further clarify the DM behavior. In interphase and early prophase cells, DMs were found to be dispersed in nuclei and did not overlap with CREST staining at all **(Fig. 4 A, B, D, E)**. In prometaphase cells, when chromosomes aligned as wheel-like structure, centromeres located inside as they were pulled inward by the attached mitotic spindles

(Fig. 4 C, F). In contrast, DMs were left behind at the nuclear periphery and they associated with distal chromosomal arms, but not necessarily with telomeric regions **(Fig. 4 C, F)**. DMs frequently formed clusters there, either big ones or small ones depending on the number of DMs. Inward relocation of centromeres and peripheral relocation of DMs appeared to take place simultaneously, while chromosome condensation occurred **(compare Fig. 4A and C)**. In metaphase cells, DMs never aligned on metaphase plates, and they associated to the periphery of the aligned chromosomes **(Fig. 4 G, J)**. In anaphase cells, while sister chromatid were pulled apart by the attached spindle microtubules, sister minute chromosomes stayed together and still associated with distal chromosomal arms **(Fig. 4 H, K)**. In telophase cells, most of the DMs were found to be incorporated into daughter nuclei while a few DMs were incorporated into independent entities called micronuclei **(Fig. 4 I, L)** (Shimizu et al., 1996). Throughout mitosis, no interaction between mitotic spindle and DMs was observed **(Fig. 4 E, F, J, K, L)**, providing the first direct evidence that DMs do not associate with microtubules due to their lack of centromeres.

Intracellular localization of EBNA-1 protein

The DMs with the integrated lac operator repeats are actually chimeric molecules, which consist of native DMs and multiple copies of the integrated EBV-lacO vectors. Not only lac operator repeats but also oriP sequence and EBNA-1 gene should be on the recombined extrachromosomal chromatin bodies. We checked the expression level of EBNA-1 protein by western blotting and found that the established clones indeed expressed EBNA-1 **(Fig. 5 top)**. Therefore, we determined the intracellular localization of EBNA-1 protein using immunofluorescence. EBNA-1 protein should bind to the oriP sequences which have been integrated into recombined DMs. Indeed, we found that approximately 45% of the cells with lacR-GFP labeled DMs had distinct foci of EBNA-1 overlapping DMs **(Fig. 5D, E)**. However, the same experiment also revealed that approximately 50% of the cells with labeled DMs did not show any detectable EBNA-1 foci **(Fig. 5D, F)**. Curiously, a few cells with labeled DMs (~3%) showed intense and diffuse nuclear staining of EBNA-1 (data not shown). Independent of the presence and the absence of EBNA-1 foci, lacR-GFP labeled DMs always showed peripheral relocation during prophase and chromosome

tethering (**Fig. 5B, C**). Therefore, heterogeneous EBNA-1 expression appeared to have little effect, if any, on the behavior of recombined DMs. Heterogeneity of EBNA-1 expression may be due to the disruption of the EBNA-1 gene which occurred during the vector integration into DMs.

Real time observation of DM behavior using dual-color GFP labeling

In order to observe the behavior of EBV-integrated DMs in living cells, we took advantage of in vivo expression of GFP fusion proteins. Different color versions of GFP protein, cyan (CFP) and yellow (YFP), has been used for dual-color labeling and real-time observation (Ellenberg et al., 1999), and we have applied the technology to differentially label entire chromosomes and DMs. Retroviral vectors expressing either H2B-CFP or lacR-YFP were prepared for this purpose. H2B-CFP is expected to label chromosomes, including DMs, as previously described (Kanda et al., 1998), while lac repressor-YFP should label only DMs as descried above. Cells expressing both constructs were obtained by simultaneous infection of two different retroviruses. We can distinguish chromosomes and DMs in the same cell with minimal to no spectral overlap using a microscope equipped with filter sets specific for CFP and YFP detection (**Fig. 6A**). Using this system, we successfully observed the mitotic behavior of DMs in living cells (**Fig. 6B, C**). In Fig. 6B, a big cluster of DMs were pulled by extended chromosomal arms of both groups of segregating chromosomes, resulting in uneven distribution into daughter nuclei. In Fig. 6C, paired dots of DMs were observed at the nuclear periphery in late prophase (time 06), and clustering and chromosome tethering of DMs were clearly observed in anaphase (time 36). In early G1 phase, some of the DMs were still observed as paired dots, demonstrating the nondisjunction of sister minute chromosomes (time 88). DMs continued to be at the nuclear periphery for a while (time 104), but they gradually dispersed throughout nuclei and they were no longer identified as paired dots (time 150). These results demonstrate that chromosome tethering of DMs contributes for their efficient incorporation into daughter nuclei. In this point of view, cellular and viral extrachromosomal molecules behave similarly so that they can minimize their catastrophic loss while nuclear membrane breaks down during mitosis.

Discussion

In this study, we used the lac operator/lac repressor-GFP system (Robinett et al., 1996) to track the behavior of the transfected EBV vectors in human cells. This strategy offers significant advantages over FISH protocols for detecting the localization of transgenes. Transgenes containing lac operator repeats can be detected by lacR-GFP staining immediately after simple fixation. Moreover, nuclear and chromosomal structures can be preserved better using the lacR-GFP system compared to FISH, since the latter requires denaturation of chromosomal DNA which results in blurring of fine chromosomal structures (Robinett et al., 1996).

EBV-lacO vectors transfected into HeLa cells were found to associate with mitotic chromosomes. By contrast, the same vector lacking EBNA-1 gene was scattered around the cytoplasm and did not associate with mitotic chromosomes, which corresponded well with its poor transformation efficiency after drug selections. Mutants of extrachromosomally maintained viral vectors, specifically BPV vectors, which fail to tether to mitotic chromosomes have never been detected by FISH as they are most likely washed away during hybridization/washing protocol required for FISH (Ilves et al., 1999). Therefore, our observation represents the first cytological evidence that deleting EBNA-1 can disrupt the chromosome tethering of the EBV vectors. Although it has long been known that oriP and EBNA-1 are the minimal elements required for stable episomal maintenance of EBV vectors (Yates et al., 1985), the role of EBNA-1 in this process remains obscure. Our observation has highlighted that not only efficient replication but also efficient transmission of replicated molecules to daughter cells can be accomplished by EBNA-1. Chromosome tethering of oriP vectors, mediated by EBNA-1, can greatly increase the efficiency of their re-incorporation into daughter nuclei, as has been proposed recently (Aiyar et al., 1998). EBNA-1 may mediate the association between oriP vectors and chromatin in interphase nuclei as well, although we cannot microscopically observe the association as chromatin fibers are decondensed in interphase nuclei. If this is the case, chromatin-associated oriP vectors can efficiently use the same replication machinery as the one which chromosomal replication origins use

during S phase. This may explain why the replication of EBV vectors is strictly under control of cellular replication program (Yates and Guan, 1991).

The transfected EBV-lacO vectors were extrachromosomally maintained in HeLa cells and did not show any evidence of chromosomal integration even after drug selection (data not shown). However, when the same vector was transfected into COLO320DM cells harboring DMs, drug resistant clones in which the vector specifically recombined with DMs were obtained with very high frequency. We did not see any integration of the EBV-lacO vectors into normal chromosomes in these clones. EBV-mediated DM specific labeling was successfully applied to another cancer cell line, CRL2270 neuroblastoma line (data not shown). The results demonstrate that EBV-lacO vectors remain as independent extrachromosomal molecules in cells without DMs, while there is a high probability of integration of multiple copies into DMs.

Successful introduction of lac operator repeats into DMs enabled us to rapidly examine the behavior of the chimeric extrachromosomal molecules not only in fixed cells but also in living cells. We used this strategy and clarified the sub-nuclear choreography of EBV-integrated DMs during mitosis. Specifically, their peripheral relocation in prophase, nondisjunction of sister minutes, chromosome tethering throughout mitosis, and redistribution in early G1 phase were observed. Our observation of H2B-GFP labeled DMs without EBV-integration showed similar chromosome tethering (Kanda et al., 1998), indicating integration of EBV vectors did not affect the DM behavior. Chromosome tethering of DMs resembles to that of EBV vectors, which requires cis-acting oriP sequence and trans-acting EBNA-1 protein. Therefore, an interesting possibility is that repeated arrays of amplicons on DMs may somehow act as cis-acting sequences to which cellular trans-acting factor(s) can bind and mediate the association with mitotic chromosomes. One significant difference between DMs and EBV vectors is that most DMs associate with distal chromosomal arms while EBV vectors appear to randomly associate with mitotic chromosomes. DMs linked to distal chromosomal arms were previously observed by light microscopy (Levan and Levan, 1978). In addition, electron-microscopic observation pointed out that DMs frequently associate with telomeric regions (Hamkalo et al., 1985; Jack et al., 1987). However, we clearly saw some DMs associating

with chromosomal arms but not with telomeric regions, arguing against the possibility that DMs are specifically associating with telomeres. Rather, we speculate that DMs, but not EBV vectors, are subjected to antipolar force to which chromosomal arms are known to be subjected (Fuller, 1995). Such antipolar force would push DMs away from the spindle poles, while putative interacting force would try to keep DMs associated with mitotic chromosomes.

Our strategy has also provided direct evidence of the nondisjunction of sister minute chromosomes until early G1 phase, which was previously demonstrated using the premature chromosome condensation protocol (Takayama and Uwaike, 1988). Electron microscopic analyses have shown that sister minutes are connected by extended chromatin fibers, suggesting that they are catenated (Barker and Stubblefield, 1979; Hamkalo et al., 1985; Jack et al., 1987; Rattner and Lin, 1984). Therefore, nondisjunction can be due to the failure of decatenation of the sister minutes. Alternatively, repeated arrays of amplicons in DMs may have configuration that favor nondisjunction of sister minutes and clustering of DMs, which may also contribute for chromosome tethering of DMs.

The precise mechanism of targeted integration of EBV vectors into multiple DMs remains unknown. It has been reported that two different DMs recombined each other to make larger ones (Coquelle et al., 1998), demonstrating that DMs are highly recombinogenic compared to normal chromosomes. Therefore, one interpretation is that, although EBV vectors randomly associate with normal chromosomes as well as DMs, they only recombine with DMs as DMs are more recombinogenic. Equally likely possibility is that EBV vectors prefer to occupy the same intranuclear territory as DMs in interphase nuclei. If this is the case, EBV vectors may preferably share replication and/or transcription machinery with recombinogenic DMs, which may elevate the probability of their recombination. Our observation that the vector lacking EBNA-1 randomly integrated into normal chromosomes as well as into DMs supports the latter idea. In either case, it is likely that the recombination between DMs and the EBV vector occurred early during drug selection. Once a recombined extrachromosomal molecule, containing a blasticidin resistance gene, is generated, the recombined molecule can accumulate to a high copy number by uneven segregation

during mitosis under continuous selective pressure of blasticidin. LacO-labeled DMs will gradually replace original DMs, and eventually cells having only lacO-labeled DMs would appear.

We did not see any significant change of DM behavior caused by EBV integration into DMs. However, we cannot completely exclude the possibility that EBNA-1 protein affects the DM behavior through their ability to link DNA molecules. To minimize the possible artifact caused by EBNA-1 expression, we also examined whether transient EBNA-1 expression, expressed from an independent vector, can target oriP plasmid into DMs. We found that, although EBNA-1 expression, estimated by western blotting, was minimal in the established cell lines, specific recombination between DMs and oriP plasmids still occurred (data not shown). Using these cell lines, similar intracellular behavior of DMs were observed using lacR-GFP staining, strongly suggesting that EBNA-1 expression had little effect, if any, on DM behavior.

Finally, as we now know that at least three different extrachromosomally replicating viruses, EBV, BPV and KSHV, tether to mitotic chromosomes, it is tempting to speculate that these viruses may target common chromosomally-associated protein(s) for their tethering. Recently, EBNA-1 domain which is required for mitotic chromosome binding has been identified using a series of deletion mutants of EBNA-1 fused to GFP (Marechal et al., 1999). Our strategy will be used to re-examine various EBNA-1 mutants for their ability to link oriP plasmids and mitotic chromosomes. The same strategy can be used for analyzing other extrachromosomally replicating viruses as well. The results will give us an important clue for identifying the putative chromosomal target protein of the viral proteins. Furthermore, an intriguing possibility is that viral and cellular extrachromosomal DNAs shares a common machinery for their chromosome tethering. If there exists chromosomal protein(s) to which EBNA-1 binds, it would be interesting to test the possibility that the same chromosomal protein(s) act in trans to keep DMs associated with mitotic chromosomes.

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Figure legends

Fig. 1 Experimental system for tracking transfected EBV vectors.

(left) The EBV-lacO vector contains EBNA-1 gene and the latent replication origin oriP. OriP contains total 24 copies of EBNA-1 binding sites clustered in two functional elements (see text). Bi-directional replication initiates from the dyad symmetry element. The vector has subcloned 256 tandem repeats of lac operator, to which lac repressor-GFP fusion protein binds with high affinity. The vector also has blasticidin resistance gene (driven by SRα promoter) as a drug selection marker.

(right) A splicing retroviral vector used for the expression of lac repressor-GFP fusion protein. For translation, the primary transcript is spliced (indicated by the bent arrow) using native retroviral splice-donor (SD) and -acceptor (SA) sequences. A nuclear localization signal added to the lac repressor GFP gene is shown as a black box.

Fig. 2 The EBV-lacO vectors associate with mitotic chromosomes in HeLa cells.

HeLa cells stably expressing lac repressor-GFP protein (A) were transfected with either the EBV-lacO vector (B, C, D, and E) or the vector lacking EBNA-1 gene (F). Mitotic cells were collected 6 days after transfection, and chromosomes were counter-stained with Hoechst 33342 (blue). Transfected vectors were identified as fluorescent dots (green).

Fig. 3 DMs are preferred targets for EBV vector integration

(A) Two color FISH analyses demonstrating the colocalization of lac operator repeats with DMs. Biotinylated c-myc cosmid and digoxigenin labeled lac operator repeat were used for hybridization. Signals were detected by FITC-avidin (for c-myc: green) and rhodamine-conjugated anti-digoxigenin (for lactose operator repeat: red). Chromosomes were counter-stained with DAPI (blue). Intrachromosomal c-myc loci are shown by arroheads in the merged image (right).

(B) Frequency of colocalization of DMs and lac operator repeats. Fifty metaphase spreads were examined for the colocalization, and they were divided into three categories; complete overlap (left),

partial overlap (middle), and no overlap (right). The numbers of metaphase spreads of each category are shown.

(C) Two color FISH analyses using chromatin fibers prepared from untransfected COLO320DM cells (top) and cells transfected with the EBV-lacO vector (bottom). Note the lac operator signals (red) and c-myc signals (green) on the same chromatin fibers (DAPI: blue).

(D) Genomic DNAs of parental COLO320DM cells (lanes 1 through 3, ten copies and 1 copy equivalent of EBV-lacO plasmids added in lanes 1 and 2, respectively) and lac operator integrated cells (lane 4) were digested with HindIII and analyzed by Southern blotting using a probe specific to lac operator repeats. An arrow indicates the expected size of the lac operator repeats.

Fig. 4 Specific labeling of DMs using lac repressor-GFP and their mitotic behavior

Cells with lac operator-labeled DMs were infected with retrovirus expressing lac repressor-GFP, and DMs were visualized as fluorescent dots (green). Infected cells were processed for immunofluorescence analyses while preserving the fluorescence of lac repressor-GFP. Chromosomes were counterstained with DAPI (blue). Centromeres were stained with CREST antiserum and a Cy5-conjugated secondary antibody (red in A, B, C, G, H, and I), while microtubules in the same cells were stained with anti α-tubulin antibody and a rhodamine-conjugated secondary antibody (red in D, E, F, J, K, and L). DMs associating with distal chromosomal arms, but not with telomeres (arrowheads), are shown by arrows (C and F). DMs being incorporated into micronuclei are shown by arrows (I and L).

Fig. 5 EBNA-1 protein made distinct foci which coincided with EBV-integrated DMs

(top) EBNA-1 expression in the established ell lines. Whole cell extracts were prepared from parental COLO320DM cells (lane 1) and three independent clones of EBV-lacO transfected cells (lanes 2 through 4) and processed for Western blotting using EBV-immune human serum reactive to EBNA.

(bottom) Cells with EBV-integrated DMs were processed for immunofluorescence analyses using the same EBV-immune human serum. DMs were stained with lac-repressor-GFP (green in A, B, C), EBNA-1 with a Cy5-conjugated secondary antibody (red in D, E, F). Merged images are shown in (G, H, I). Chromosomes are counterstained with DAPI (blue). Note that EBNA-1 colocalizes with DMs but not in every cell.

Fig. 6 Real time observation of DM behavior using dual-color GFP labeling

(A) CFP and YFP can be used for dual color labeling. Entire chromosomes, including DMs, are stained with H2B-CFP (left), while DMs are specifically stained with lac repressor-YFP (middle). Merged image is shown in right.

(B, C) Two different series of real time analyses of DM behavior during mitosis. Dual color images were collected every minute. Representative images at indicated time points (minute) are shown. One of the daughter nuclei went out of the focal plane in early G1 phase (time point 088 in C), when nondisjunction of sister minutes were clearly observed (shown by arrows).

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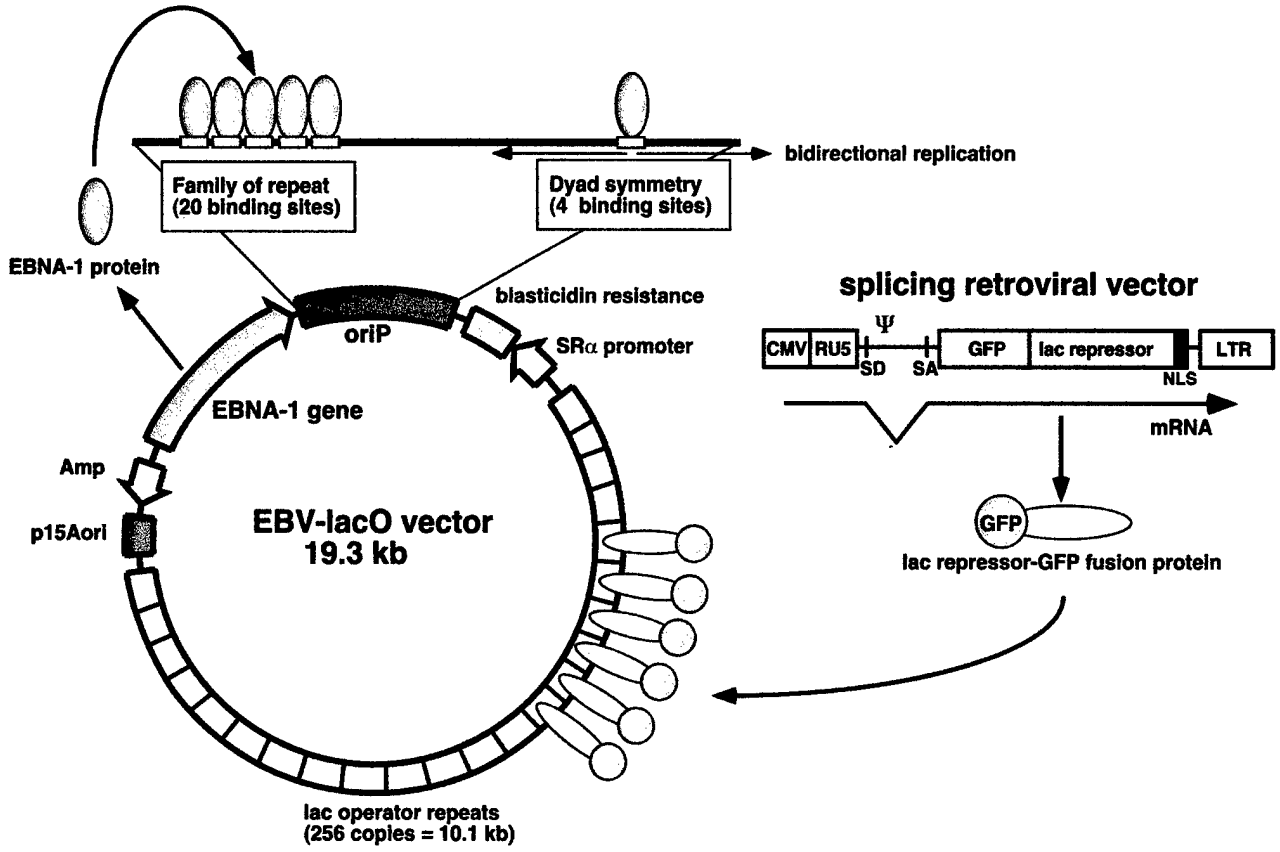
Fig. 1


bidirectional replication
Family of repeat
(20 binding sites)
Dyad symmetry
(4 binding sites)
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p15Aori
EBV-lacO vector
19.3 kb
lac operator repeats
(256 copies = 10.1 kb)
splicing retroviral vector
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Fig. 2

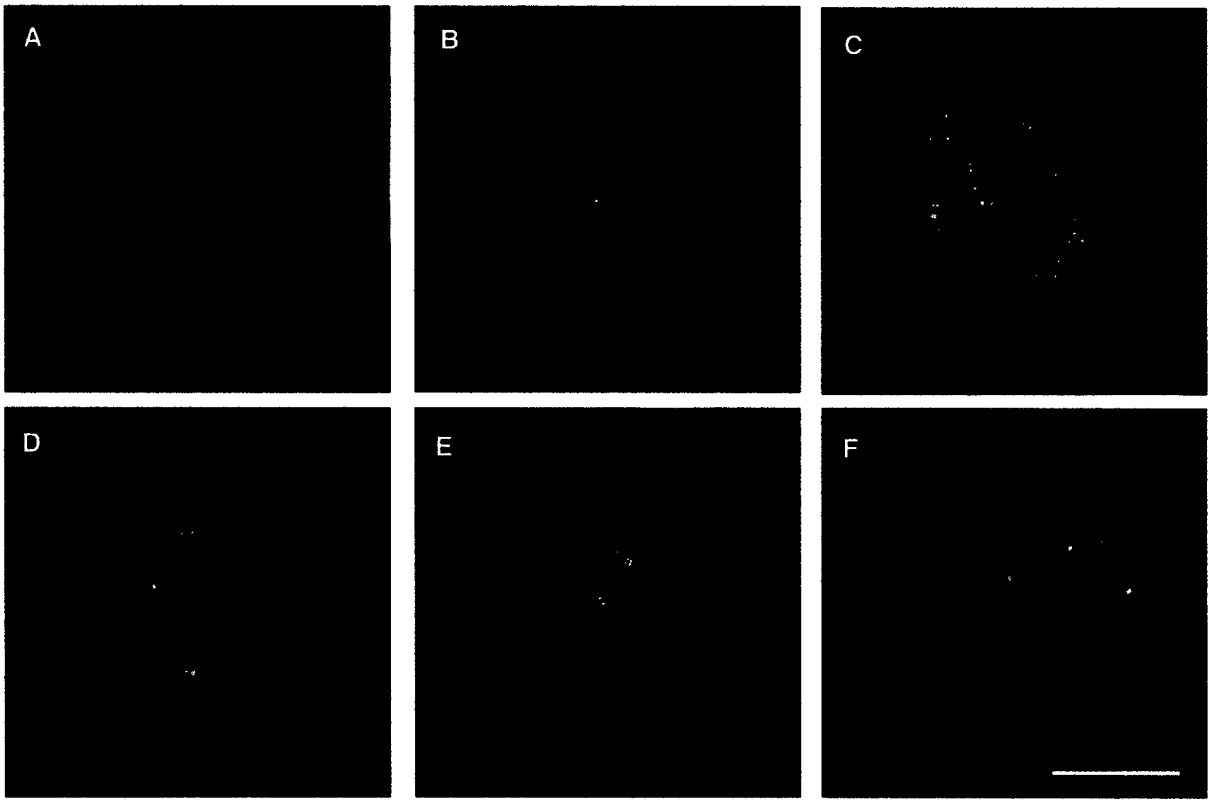
A
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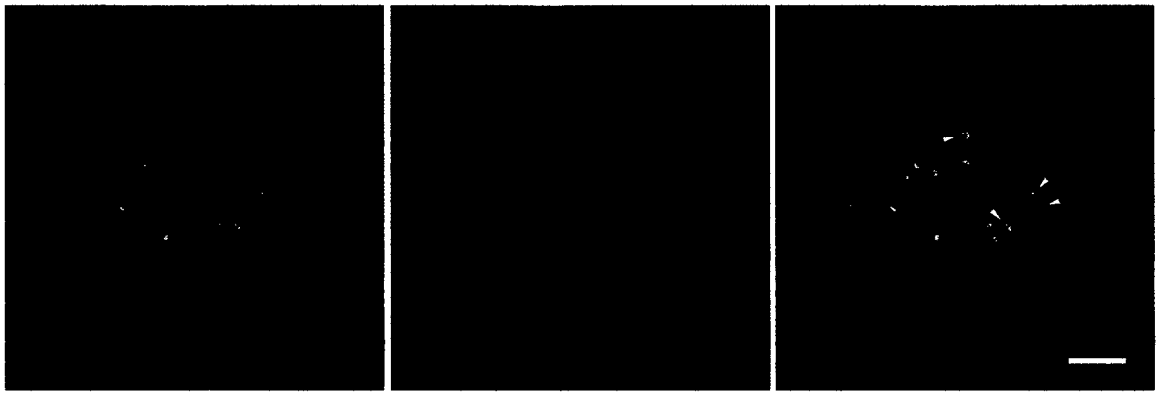
Fig. 3

A



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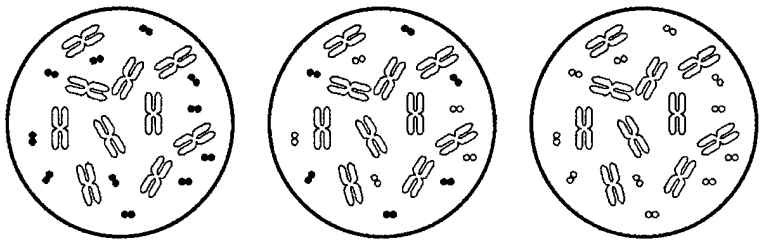

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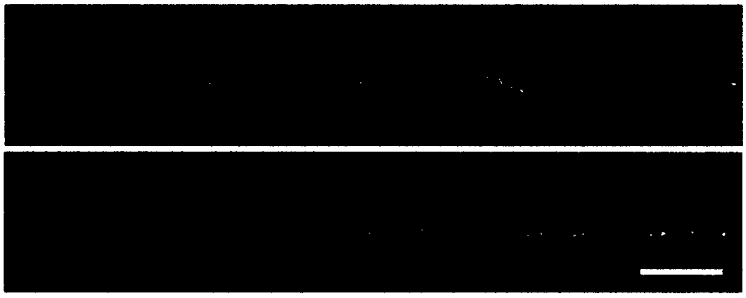

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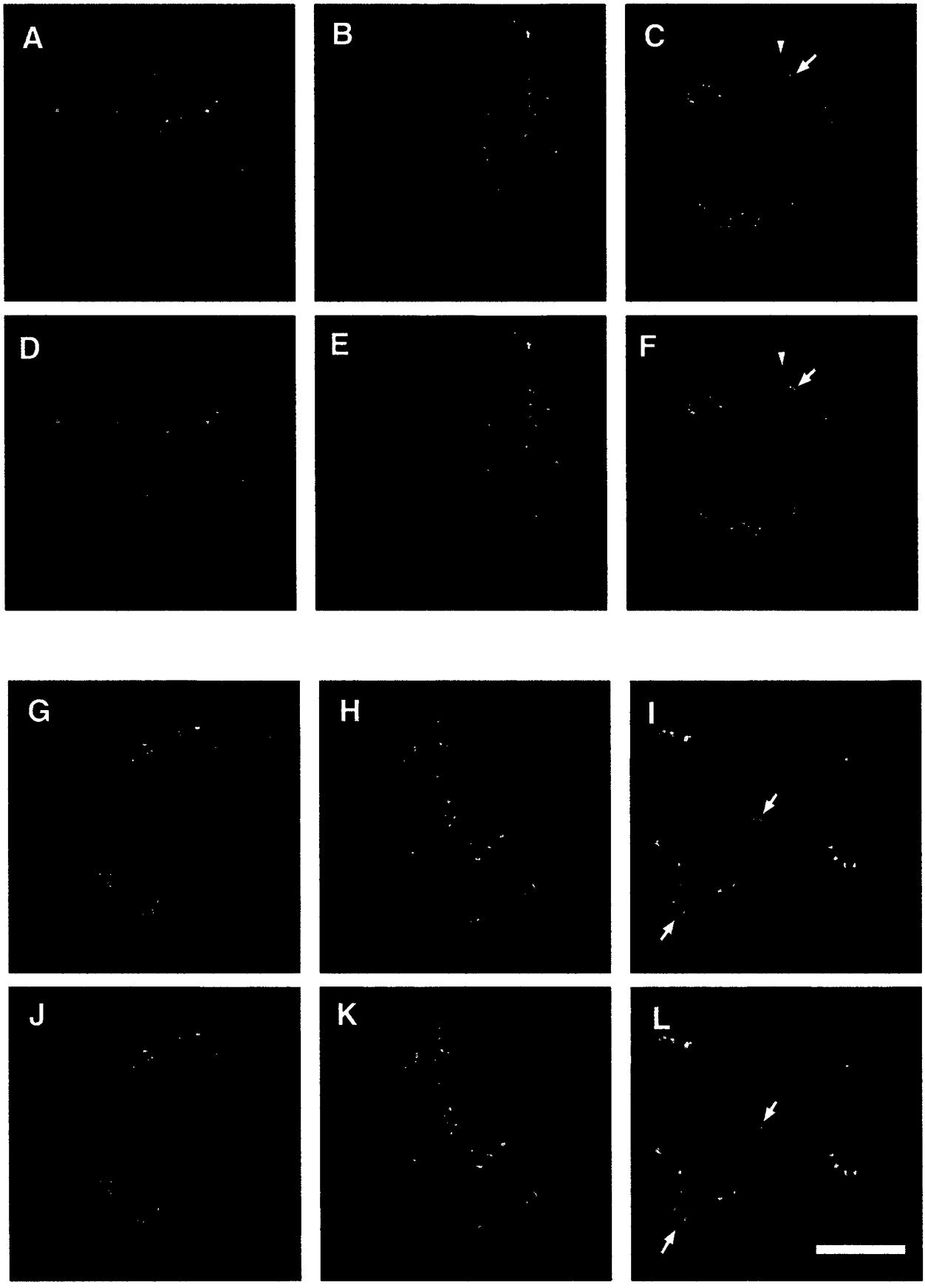
Fig. 4



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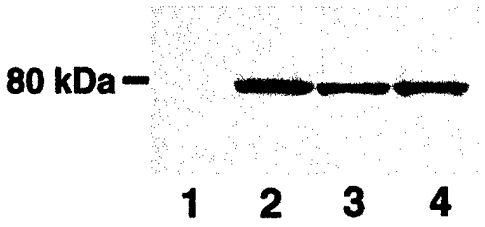


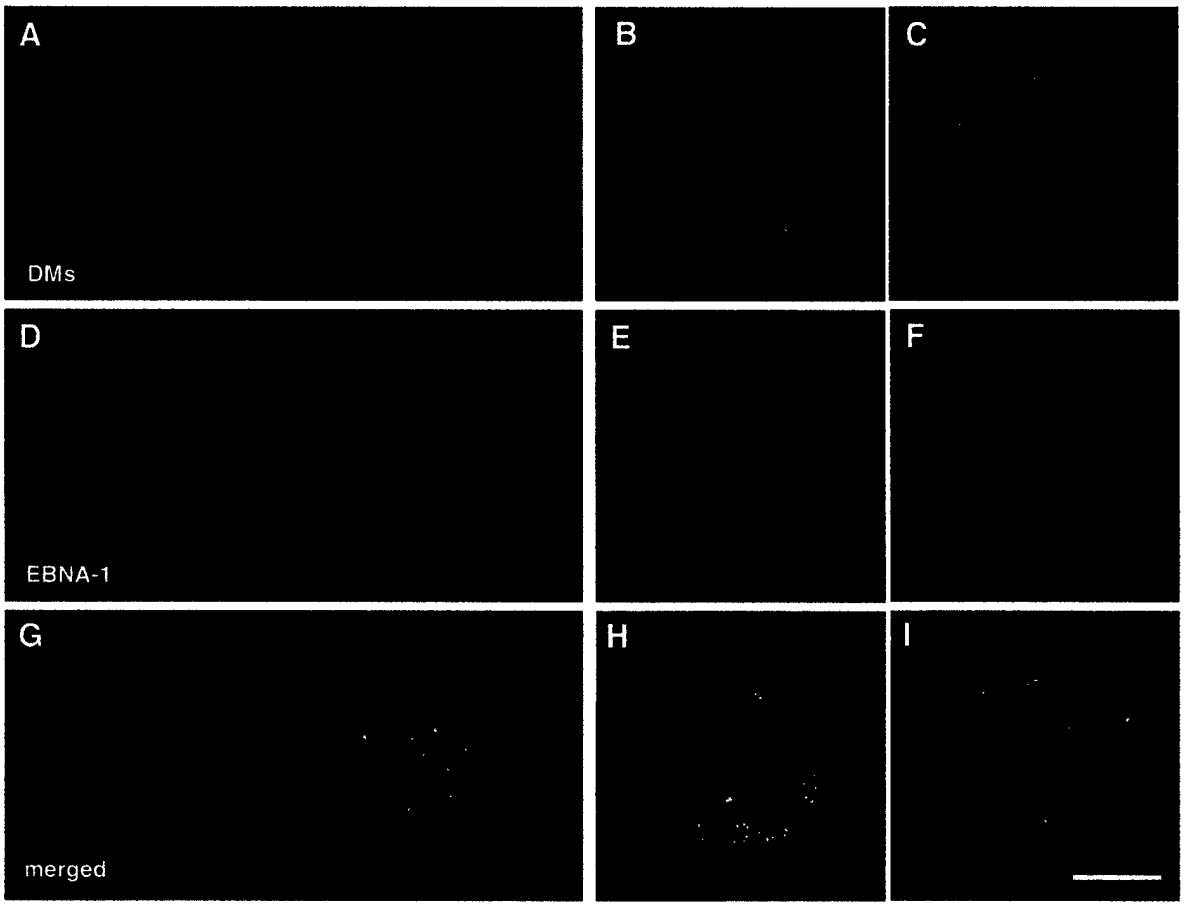
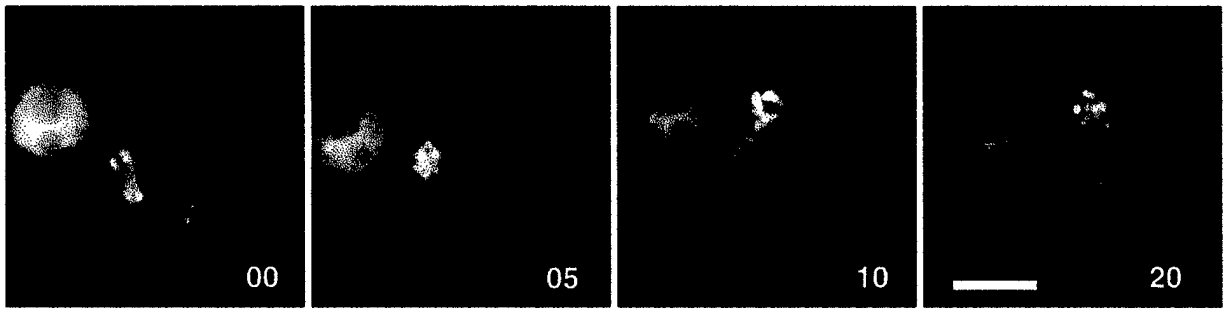


Fig. 6

A

B



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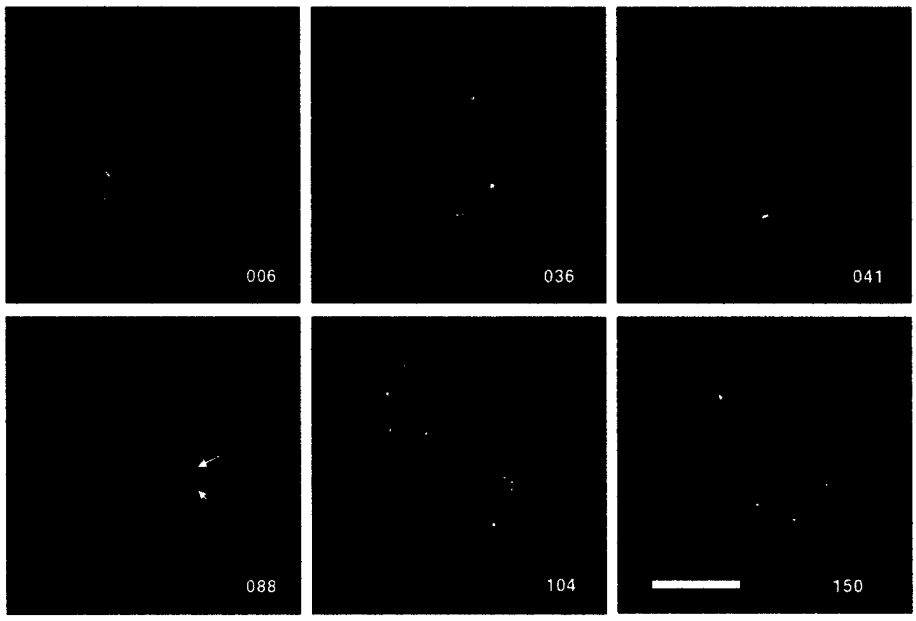

Selective Entrapment of Extrachromosomally Amplified DNA by Nuclear Budding and Micronucleation during S Phase

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Abstract. Acentric, autonomously replicating extrachromosomal structures called double-minute chromosomes (DMs) frequently mediate oncogene amplification in human tumors. We show that DMs can be removed from the nucleus by a novel micronucleation mechanism that is initiated by budding of the nuclear membrane during S phase. DMs containing c-*myc* oncogenes in a colon cancer cell line localized to and replicated at the nuclear periphery. Replication inhibitors increased micronucleation; cell synchronization and bromodeoxyuridine–pulse labeling demonstrated de novo formation of buds and micronuclei during S phase. The frequencies of S-phase nuclear budding and micronucleation were increased dramatically in normal human cells by inactivating p53, suggesting that an S-phase function of p53 minimizes the probability of producing the broken chromosome fragments that induce budding and micronucleation. These data have implications for understanding the behavior of acentric DNA in interphase nuclei and for developing chemotherapeutic strategies based on this new mechanism for DM elimination.

THE accumulation of structural and numerical chromosome abnormalities in eukaryotic cells is limited by coordinating biosynthetic and repair processes with cell cycle checkpoints (Hartwell and Kastan, 1994). Mutations in genes involved in these transactions occur commonly during cancer progression and can greatly elevate the frequencies of base alterations or large-scale chromosome rearrangements. For example, defects in cell cycle–control pathways involving the p53 tumor suppressor gene create a permissive environment in which cells with aneuploidy, chromosome translocations, and gene amplification arise at high frequency in response to stresses created by antimetabolites or oncogene overexpression (Livingstone et al., 1992; Yin et al., 1992; Denko et al., 1994).

The types of aberrant chromosomal structures generated in cells with defective repair and cell cycle control functions are likely to be constrained by nuclear structure. For example, chromosomes with very long arms tend to generate nuclear projections variously referred to as "blebs" or "buds" (Ruddle, 1962; Lo and Fraccaro, 1974; Toledo et al., 1992; Pedeutour et al., 1994). A recent study in peas demonstrated that excessive DNA within a single chromosome arm generated a nuclear projection that was cut when the cell division plate formed after telophase (Schubert and Oud, 1997). Sequences enclosed in such projections are often detected in micronuclei, suggesting that projections can be precursors of micronuclei (Toledo et al., 1992; Pedeutour et al., 1994), and that the chromosomal sequences they contain can be lost from the nucleus. These data indicate that a maximum allowable size exists for each chromosome arm within the nuclei of specific cell types.

Circular, autonomously replicating DNA fragments such as double-minute chromosomes (DMs)[1] are also frequently generated in cancer cells (Barker, 1982; Cowell, 1982; Benner et al., 1991). These structures encode proteins that provide survival advantages in vivo, or resistance to a variety of chemotherapeutic agents in vitro (Alitalo and Shwab, 1986; Wahl, 1989; Von Hoff et al., 1992; Brison, 1993; Shimizu et al., 1994; Eckhardt et al., 1994). DMs replicate using cellular replication origins (Carroll et al., 1993), but lacking centromeres, they do not segregate by the same mechanisms used by chromosomes. Consequently, DMs are lost spontaneously in the absence of selection. Drugs such as hydroxyurea (HU) significantly increase the loss rate of DMs in human and rodent cell lines (Snapka and Varshavsky, 1983; Von Hoff et al., 1991; Von

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1. *Abbreviations used in this paper*: BrdU, 5-bromo-2′-deoxyuridine; DAPI, 4′-6-diamidino-2-phenylindole; DM, double-minute chromosome(s); FISH, fluorescence in situ hybridization; HU, hydroxyurea; PALA, *N*-phosphoracetyl-L-aspartate; PFA, paraformaldehyde; PI, propidium iodide; RPE-h, normal human retinal pigmented epithelial cells.

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Hoff et al., 1992; Eckhardt et al., 1994; Canute et al., 1996). DM elimination results in increased drug sensitivity, reduced tumorigenicity, or differentiation, depending on the proteins expressed by DM-encoded genes (Snapka and Varshavsky, 1983; Snapka, 1992; Von Hoff et al., 1992; Eckhardt et al., 1994; Shimizu et al., 1994). Identifying the mechanisms by which DMs are eliminated could enable the development of new and more selective chemotherapeutic strategies, since DMs are uniquely found in cancer cells, and chromosome loss should not be induced by such treatments.

Like abnormally long chromosome arms, DMs have also been reported to be preferentially incorporated within micronuclei that are removed from the cell (Von Hoff et al., 1992; Shimizu et al., 1996). It is clear that small size alone does not guarantee selective enclosure of DNA fragments within micronuclei because a centric minichromosome the size of a typical DM is effectively excluded from micronuclei (Shimizu et al., 1996). This observation is consistent with the classical mechanism of micronucleus formation that involves the enclosure of lagging acentric chromosome fragments as nuclear membranes reform at the end of mitosis (Heddle and Carrano, 1977; Heddle et al., 1983). Thus, one would expect postmitotic enclosure of DMs within micronuclei since they typically lack functional centromeres (Levan et al., 1976). However, DMs appear to associate with chromosomes or nucleoli, which may enable most of them to evade such a postmitotic mechanism. The ability of DMs to "hitchhike" by association with mitotic chromosomes or nucleoli provides one explanation of why few micronuclei were detected at the midbody in a cell line containing numerous DMs (Levan and Levan, 1978), and their surprisingly efficient partitioning to daughter cells in some cell lines (Levan and Levan, 1978; Hamkalo et al., 1985). However, the interphase behavior of normal chromosomes and DMs may differ because DMs lack the centromeres and/or telomeres that position chromosomes in restricted territories and produce a choreographed set of chromosome movements during S phase (DeBoni and Mintz, 1986; Cremer et al., 1993). It has neither been determined whether acentric DM–DNA occupies positions different from chromosomes in interphase, nor whether this could enable their removal from the nucleus by a budding process like that observed for abnormally long chromosomes (Ruddle, 1962; Jackson and Clement, 1974; Lo and Fraccaro, 1974; Miele et al., 1989; Toledo et al., 1992).

DMs provide an excellent model for analyzing the nuclear behavior of multimegabase replicons lacking a centromere and telomeres. We show that DMs can preferentially localize the nuclear periphery, whereas chromosomally amplified sequences occupy a more central position. Although the micronuclei that entrap acentric chromosome fragments have typically been viewed to be generated postmitotically, we provide evidence for a novel micronucleation mechanism that involves the formation of nuclear projections which we refer to as buds. Buds form during S phase and appear to selectively associate with DMs replicating near or at the nuclear periphery. Since micronuclei are indicators of DNA damage and are produced at much higher frequencies in tumor cells than in normal cells, we investigated whether micronucleation frequency is increased in cells with defects in cellular responses to DNA damage. We show that loss of p53 function increases the frequency of micronucleation and enables buds and micronuclei to be produced under conditions expected to lead to chromosome breakage.

Materials and Methods

Cell Culture

Human COLO 320DM (CCL 220) and COLO 320HSR (CCL 220.1) neuroendocrine tumor cells were obtained from American Type Culture Collection (Rockville, MD) and then single cell subclones were obtained by limiting dilution (Von Hoff et al., 1988). The locations of amplified c-*myc* genes to DMs or HSRs were confirmed by fluorescence in situ hybridization (FISH) using c-*myc* cosmid DNA. The cells were grown in RPMI 1640 medium supplemented with 10% FBS. The WS1 human embryonic skin fibroblast, obtained from American Type Culture Collection (CRL 1502), was cultured in DME supplemented with 10% heat-inactivated, dialyzed FBS, and 1× MEM nonessential amino acids. WS1-neo and WS1-E6 were kind gifts of S. Linke (National Institutes of Health, Bethesda, MD), and were generated by infecting WS1 with retroviral vectors expressing genes encoding either neomycin resistance or both neomycin resistance and the E6 protein from human papilloma virus 16, respectively (Linke et al., 1996). RPE-h (normal human retinal pigmented epithelial cells) and its neo and E6 derivatives were also kindly provided by S. Linke and the parental cells were obtained from Cell Genesys, Inc. (Foster City, CA). Epithelial cells were cultured in the same way as WS1.

Chemicals

Aphidicolin, 5-bromo-2′-deoxyuridine (BrdU), coumarin (1, 2-benzopyrone), deferoxamine mesylate (desferrioxamine mesylate), DMSO, hydroxyurea, nicotinamide, thymidine, and nocodazole (methyl-[5-[2-thienylcarbonyl]-1H-benzimidazol-2-YL]carbamate) were obtained from Sigma Chemical Co. (St. Louis, MO). Guanazole (3,5-diamino-1,2,4-triazole) was from Aldrich Chemical Co. (Milwaukee, WI). PALA (*N*-phosphonacetyl-L-aspartate) was provided by the Drug Biosynthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD).

Cell Cycle Analysis

Cell cycle distribution was analyzed using flow cytometry as described previously (Yin et al., 1992; Di Leonardo et al., 1994). Cells treated with the indicated concentrations of drugs for the indicated times were labeled with 10 μM BrdU for 30 min. The cells were collected, fixed with 70% ethanol, treated with 0.1 N HCl containing 0.5% Triton X-100 (Mallinckrodt, Paris, KY), and then followed by boiling for 10 min and rapid cooling to denature the DNA. The nuclei were then incubated with FITC-conjugated anti-BrdU antibodies (Boehringer Mannheim Biochemicals, Indianapolis, IN) and counterstained with 2 μg/ml of propidium iodide (PI) containing RNase (200 μg/ml). Samples were analyzed using a Becton Dickinson FACScan™ (Sparks, MD). 10,000 events were collected for each sample. Data were analyzed using Sun Display as described previously (Yin et al., 1992; Di Leonardo et al., 1994).

Quantification of Micronuclei

Micronuclei containing DM sequences in COLO 320DM cells (see Figs. 2 and 3) were detected by preparing chromosome spreads using standard hypotonic swelling conditions (Lawce and Brown, 1991), followed by hybridization with a biotinylated c-*myc* cosmid probe as described previously (Shimizu et al., 1996). Total micronuclei (see Fig. 3) were determined by staining chromosome spreads with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.; 1 μg/ml in VectaShield, Vector Labs, Inc., Burlingame, CA). The adherent cells (WS1, RPE-h, and their derivatives) were grown on coverslips, fixed with cold acetone (−20°C for 5 min) followed by cold methanol (−20°C for 5 min), rehydrated with PBS, and then stained with DAPI (1 μg/ml in VectaShield). The numbers of total or DM-enriched micronuclei were scored using 60 or 100× objectives and a fluorescence microscope equipped with appropriate epifluorescence filters (model Zeiss WL; Carl Zeiss, Inc., Thornwood, NY). The results are expressed as *Frequency of Micronuclei (%)* relative to the number of interphase nuclei scored (≥1,000 for each point).

Cell Synchronization

Synchronization was performed as described previously (Stein et al., 1994). Rapidly growing COLO 320DM cells were first arrested in early S phase using excess thymidine (2 mM) for 17 h. The cells were then washed with growth medium, released into growth medium containing 25 μM 2′-deoxycytidine for 12 h (to reverse thymidine toxicity), and then incubated in 2.5 μg/ml aphidicolin for 17 h to arrest cells as they entered S phase. The arrested cells were washed with growth medium and then either released into medium lacking drug, or into medium containing nocodazole (0.4 μg/ml). Cell cycle progression was monitored using incorporation of [^{3}H]thymidine (Stein et al., 1994). To monitor the progression through mitosis, a portion (1 ml) of culture was fixed by paraformaldehyde (PFA; 2%) and then stained with DAPI. The frequency of cells in mitosis was scored using fluorescence microscopy. WS1-E6 cells were synchronized by seeding them at low density in 15-cm dishes with or without coverslips (18 × 18 mm). The day after subculture, the medium was removed and then replaced with medium containing 0.1% FCS, and then followed by the culture for an additional 48 h. Cells arrested at G0 by serum deprivation were released into growth medium containing 5 μg/ml aphidicolin for 15 h to arrest them at the beginning of S phase. Cells were released into S phase by replacing the medium with fresh growth medium lacking drug. Progression into S phase was monitored by the incorporation of [^{3}H]thymidine (Stein et al., 1994). The number of cells that were in S phase just before release from synchrony was monitored by BrdU–pulse labeling (30 min) followed by confocal examination of the labeling pattern as described in the following section (see Simultaneous Determination of DM Location and DNA Replication Using FISH and BrdU Incorporation). Concurrently, coverslips were removed, fixed with acetone and methanol, stained by DAPI, and then the frequency of micronuclei, nuclear budding, and mitotic cells were scored as described above.

Terminal Deoxynucleotidyl Transferase-mediated dUTP-Biotin Nick-end Labeling Assay

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay was done according to a previously published procedure (Gavrieli et al., 1992) modified as described below. In brief, COLO 320DM cells were fixed in 2% PFA (10 min at room temperature) and then centrifuged onto a glass slide using a cytospin (Salk Institute Shop) apparatus. The cells were further fixed in cold methanol (−20°C, 5 min) followed by cold acetone (−20°C, 5 min). The slides were rehydrated in PBS and then equilibrated in reaction buffer (200 mM sodium cacodylate, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, pH 7.2) for 15 min at room temperature. The end-labeling reaction was done by incubating the slides with the reaction buffer containing 10 μM biotin-dUTP (Boehringer Mannheim GmbH, Mannheim, Germany), and 0.3 U/μl of terminal deoxynucleotidyl transferase (Toyobo Co., Osaka, Japan), for 60 min at 37°C. The slides were washed extensively, blocked with 20% FCS, and then the incorporated biotin was detected using FITC-conjugated streptavidin as in the protocol for FISH (see below). The slides were treated with RNase A (100 μg/ml, 37°C, for 20 min), counterstained with PI, and then observed under the conditions used for FISH.

Probe Preparation and FISH

Preparation of probe from purified micronuclei was as described (Shimizu et al., 1996), except that DNA in the purified micronuclei was directly used for biotin labeling by using the BioPrime DNA Labeling System (Life Technologies Inc., Gaithersburg, MD). C-*myc* cosmid DNA probe was prepared as described (Shimizu et al., 1996). FISH using standard methanol/acetic acid-fixed nuclei was performed as described previously (Shimizu et al., 1996). Assessments of DM localization by confocal microscopy required the following procedure to preserve the spherical shape of the nuclei. This protocol, based on that developed for human lymphocytes (Ferguson and Ward, 1992; Vourc'h et al., 1993), could not be applied directly to COLO 320DM due to severe nuclear aggregation. The modified procedure involves pelleting 10 ml of COLO 320DM cells by centrifugation at 260 *g* for 5 min, followed by complete removal of the supernatant. The cells were gently suspended in 50 μl of growth medium and then 10 ml of prewarmed (37°C) 75 mM KCl, 2 mM $CaCl_2$ was added slowly. The suspension was centrifuged immediately as described above and the supernatant was removed completely. The cell pellet was loosened gently, suspended in 1 ml of 75 mM KCl and 2 mM $CaCl_2$ at 4°C, and then followed by addition of 1 ml of 75 mM KCl, 2 mM $CaCl_2$, 0.5% Triton X-100 at 4°C. The suspension was kept on ice for 10 min, then Dounce homogenized (loose fitting pestle, 5 times, at 4°C; Fisher, Pittsburgh, PA). 1.5 vol of 5% PFA in PBS was added to the suspension and then incubated for 10 min at room temperature with occasional gentle shaking. After incubation, a 1:10 vol of 1 M Tris-HCl, pH 7.4, containing 1% BSA was added and further incubated for 10 min at room temperature with gentle shaking. The fixed nuclei were washed twice with PBS containing 1% FCS and then stored at 4°C up to 1 wk. Before FISH hybridization, the fixed nuclei were sedimented by cytospin onto poly L-lysine–coated glass slides (Matsunami Glass Ind., Ltd., Osaka, Japan). Slides were treated with RNase A (Sigma Chemical Co., 100 μg/ml in 2× SSC, 37°C for 60 min), washed once with 2× SSC for 3 min, and then followed by blocking with 3% BSA in PBS for 30 min at 37°C. Slides were incubated in 50% formamide dissolved in 2× SSC for 30 min at room temperature to enable buffer equilibration, followed by addition of the hybridization mixture containing labeled probe (prepared as for standard FISH [Shimizu et al., 1996]). The sample was covered by a coverslip, sealed completely with rubber cement, denatured at 85°C, and then hybridized using overnight incubation at 37°C. Washing and the detection of the hybridized probe were performed as described previously (Shimizu et al., 1996). In some cases, intact cells were fixed directly and then hybridized. For this purpose, the cells were cytocentrifuged onto poly L-lysine–coated slides, fixed with ethanol/acetic acid (19:1) for 3 min at −20°C, rehydrated with PBS, and then treated with 4% formaldehyde in PBS for 10 min at 4°C. The slides were then washed extensively with PBS and hybridized as described above for isolated nuclei. Images were obtained using a Bio-Rad MRC600 confocal system (Hercules, CA) on a Zeiss Axiovert 135 microscope (see Fig. 6). Most images were obtained using a 63× objective (Apochromat, 1.40, oil, Carl Zeiss, Inc.), and zoom factor two. The acquired digital images were expressed as pseudocolors and then merged using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA).

Localizing DMs in Interphase Nuclei

The locations of DMs in confocal nuclear sections were determined by measuring the distance from each of the hybridized signals to the center of the nucleus using the corresponding nuclear diameter as a unit length. Coplanar PI (DNA) and FITC (hybridized signal) images intercepting the center of the nucleus were obtained from randomly chosen nuclei. The digital images were merged using COMOS software (Bio-Rad Laboratories). The threshold value for each FITC signal was lowered until each signal, representing the domain of one or more DMs, became a single dot to enable accurate distance measurements. The distances from this dot to the center of the nucleus and the nuclear diameter were determined. The location of each signal in the nucleus was expressed by dividing the former number by the latter number. According to this expression, the center of the nucleus is 0, and the outer edge of the nuclear membrane is 1. At the same time, the intensity of each signal was measured using an arbitrary unit scale. These values were determined for every signal in each of the nuclear sections using a minimum of 100 randomly chosen nuclei for each sample. This procedure gives rise to a distribution of DM signals in each two-dimensional focal plane. The heights and widths of each nucleus were found to be approximately equal, indicating that the fixation procedure preserved a spherical nuclear morphology. We assume that the distribution of signals within each nuclear vol should correspond to the number of signals we detected at the corresponding radius in two-dimensional space. Therefore, we corrected the number of signals at each radial location to represent the number that should be present in the spherical volume corresponding to that radius.

Simultaneous Determination of DM Location and DNA Replication Using FISH and BrdU Incorporation

Rapidly growing COLO 320DM cultures were pulse labeled using 10 μM BrdU (Sigma Chemical Co.) for 1 h and then followed by immediate cell collection. The pulse-labeling period was 30 min for the experiment presented in Fig. 2, *G* and *H*. The isolation of nuclei, fixation by PFA, and FISH using purified micronuclei probe were done as described above. After FISH, BrdU incorporation was determined by incubating the slides with anti-BrdU mouse monoclonal antibody (PharMingen, San Diego, CA) at a final concentration of 10 μg/ml in PBS containing 0.1% BSA. After a 60-min incubation at 37°C, the slides were washed three times with PBS for 5 min each. The slides were then treated with rhodamine-labeled anti–mouse Ig (Boehringer Mannheim Biochemicals) at a final concentration of 10 μg/ml in PBS containing 0.1% BSA. The slides were incubated

for 60 min at 37°C and then washed with PBS three times at 5 min each. The nuclei were viewed without counter staining, using an MRC 1024 (Bio-Rad Laboratories) confocal system equipped to Axiovert 135M microscope (Carl Zeiss, Inc.), and then the acquired digital images were processed as described above.

Results

Nuclear Budding during Interphase Can Selectively Entrap DMs

The classic mechanism by which acentric chromosome fragments such as DMs are lost from cells involves their enclosure within reforming nuclear membranes subsequent to telophase (for review see Heddle et al., 1991). However, there is one report that nuclear anomalies resembling micronuclei can be generated in interphase subsequent to γ irradiation (Duncan et al., 1985). We used a cell line with amplified c-*myc* genes to assess the relative contributions of postmitotic and interphase mechanisms to DM micronucleation.

A FISH analysis of COLO 320DM, a colon cancer cell line of neuroendocrine origin, is shown in Fig. 1. A biotinylated FISH probe specific for the c-*myc* amplicon in COLO 320 cells was obtained from micronuclei purified from COLO 320DM cells (Shimizu et al., 1996). FISH analysis with this probe showed that >95% of the cells in the population contained only DMs, and the remainder contained DMs along with one intrachromosomally amplified region (Fig. 1 *A*, *arrow*). Consistent with a previous report (Levan and Levan, 1978), the DMs in the prometaphase spread (Fig. 1 *A*) do not appear to be distributed randomly since many localize to the periphery of the prometaphase ring. Peripheral nuclear localization was also observed in interphase nuclei using confocal microscopy (see below).

Analyses of exponentially growing cultures of COLO 320DM cells by a conventional FISH procedure revealed that 2.8 ± 0.9% of interphase nuclei have projections or buds. These buds can be classified into two types by FISH analysis using the probe described above. The first class (56 ± 12.2% of the nuclear buds) contained highly concentrated DM sequences (Fig. 1, *B–D* show representative buds that contain DMs; *C* shows two micronuclei and one bud). We also observed a second class of buds that were stained with PI, but did not hybridize with the DM probe. This suggests that DNA that does not hybridize with the DM probe was contained in these buds. (see Discussion). However, the selectivity for acentromeric sequences such as DMs appears to be very high. This is indicated by the nuclear bud shown in Fig. 1 *D* that contains three DNA clusters that stain with PI (the red signal represents DNA staining as these samples were first treated extensively with RNAse) and that hybridize intensely with the micro-

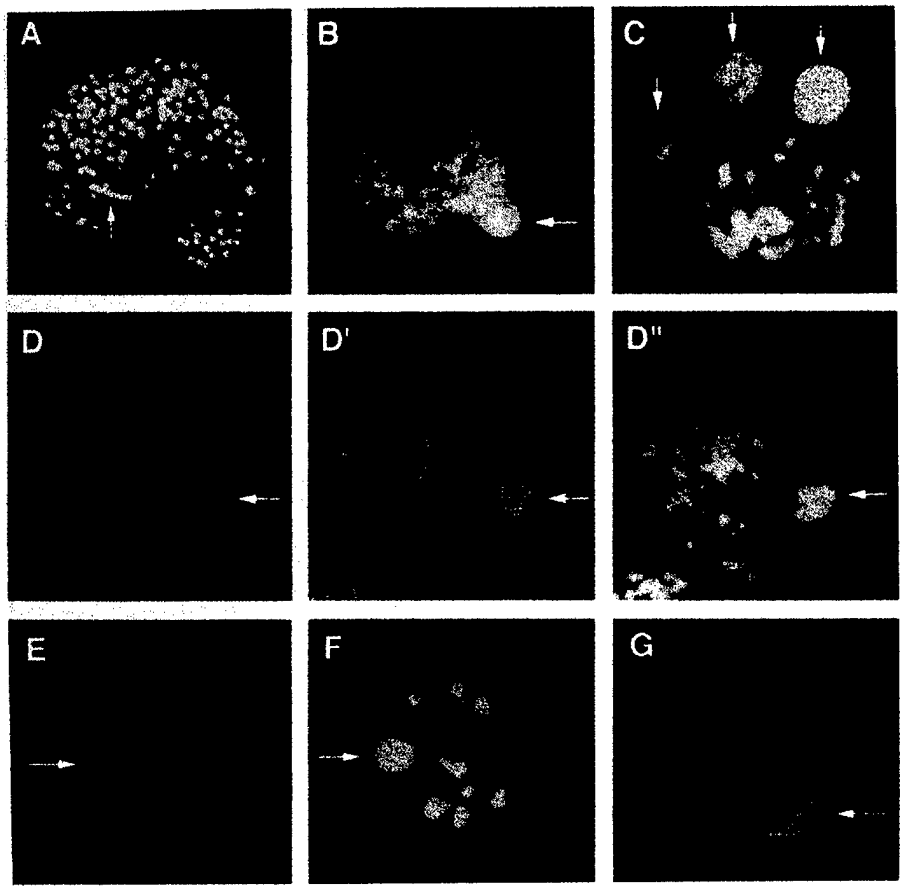

Figure 1. Nuclear budding selectively entraps DMs. Exponentially growing COLO 320DM cells were examined by a conventional FISH procedure that includes hypotonic treatment and fixation with methanol/acetic acid (3:1) (*A–E*). Alternatively, cells grown on slides were directly fixed with ethanol/acetic acid (19:1) followed by formaldehyde fixation (*F* and *G*). The slides were treated with RNase and hybridized with biotinylated DNA from purified micronuclei, except *E*, which was hybridized with biotinylated c-*myc* cosmid DNA. The hybridized probe was detected by FITC-conjugated streptavidin and then the DNA was counterstained with PI. (*A*) A prometaphase figure that shows both the specificity of the probe for DM painting and documents the peripheral location of DMs around the prometaphase chromosomes. One chromosomally integrated HSR region is indicated by an *arrow*. These DMs are selectively incorporated into the nuclear buds formed in the interphase nuclei (*B–G*, *arrows*). DM capture by buds appears to be very selective because the three dense PI-positive signals obvious in the nuclear bud (*D*) label intensely with the FITC–FISH probe (*D'*). (*D''*) The merged image.

nuclear DNA–FISH probe (Fig 1 *D'*; merge shown in *D''*). The selective inclusion of DMs into buds was also readily apparent when we used a FISH probe derived from a cosmid containing the c-*myc* gene. (Fig. 1 *E*), PFA-fixed nuclei isolated by a hypotonic method (see Fig. 6), or an isotonic method (data not shown). Furthermore, the selective inclusion of DM sequences into nuclear buds was also apparent in intact cells fixed directly by ethanol/acetic acid (19:1) followed by formaldehyde (Fig. 1, *F* and *G*). The apparent preferential inclusion of DM sequences in buds is reminiscent of our previous finding that DMs are highly enriched in micronuclei and that chromosomes or minichromosomes with functional centromeres are excluded from micronuclei (Von Hoff et al., 1992; Shimizu et al., 1996). These data suggest that buds are precursors of micronuclei.

Nuclear Buds and Micronuclei Are Formed during S Phase

The nuclei with buds exhibit a morphology typical of an interphase cell, not one in mitosis. Therefore, we determined the kinetics of formation of micronuclei and nuclear buds to ascertain whether these structures can be generated during S phase. COLO 320DM cells were synchronized at the G1/S boundary using a two-step procedure involving treatment with high thymidine concentration to arrest cells during S phase, release for 12 h to enable progression through and exit from S phase, and then incubation with the DNA polymerase inhibitor aphidicolin to arrest cells at the beginning of the next S phase (Stein et al., 1994). Removal of aphidicolin resulted in rapid entry into S phase with a peak of [^{3}H]thymidine uptake at 4 and a peak of mitosis at 10 h, respectively (Fig. 2 *A*). The cells entered a second, less synchronous cycle ≤19 h after release. The synchronization level of the first S phase was further quantified by BrdU–pulse labeling followed by confocal microscopic examination of the nuclear labeling patterns. The labeling pattern progressed as reported previously (O'Keefe et al., 1992), with six readily distinguishable patterns (Fig. 2 *G*, *Patterns 0–6*). Examination of the arrested cells ($t = 0$) revealed that 96.1% of the cells did not incorporate label (Fig. 2 *H*, *Pattern 0*) but the remaining 3.9% of cells exhibited the pattern expected for

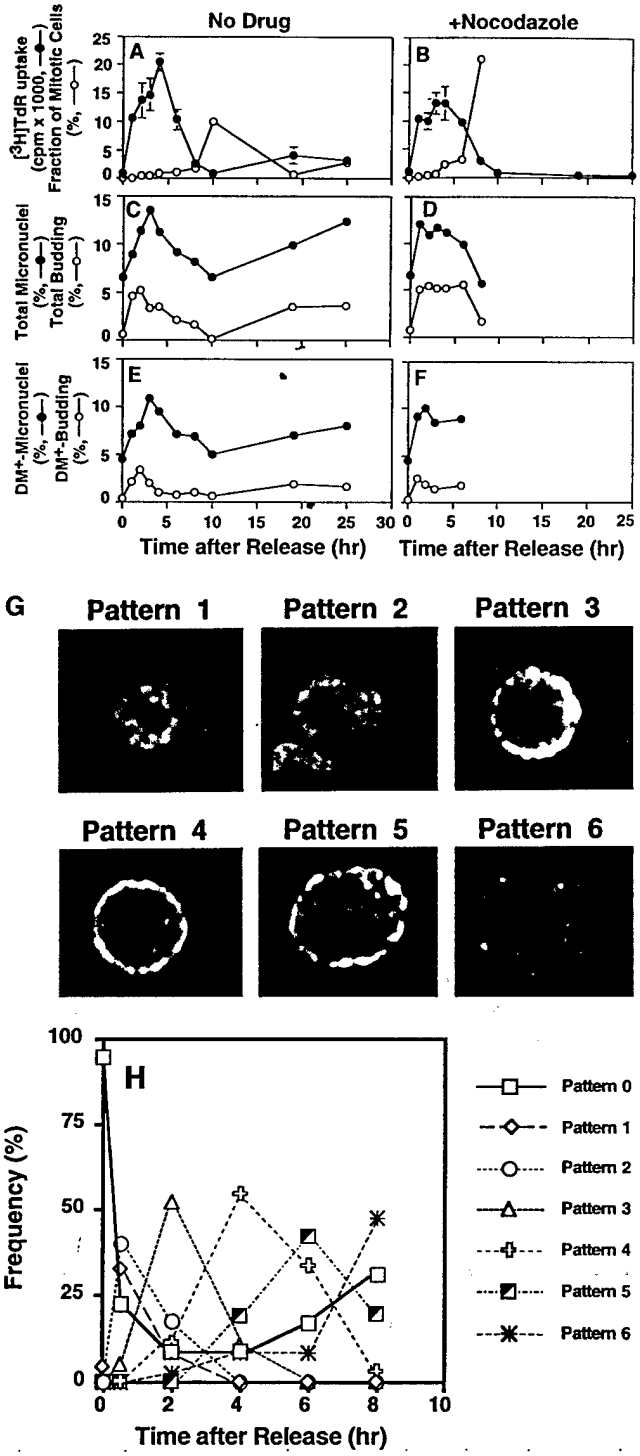

Figure 2. Formation of micronuclei and nuclear buds in a synchronized culture of COLO 320DM cells. COLO 320DM cells were synchronized at the G1/S boundary using a two-step procedure as described in Materials and Methods. The culture was divided into two portions and then released in the absence of any drug (*A*, *C*, and *E*), or the presence of 0.4 μg/ml of nocodazole (*B*, *D*, and *F*). [^{3}H]thymidine incorporation (*closed circles*) and the fraction of mitotic cells (*open circles*) were determined to monitor the synchronous progression through S and M phases, respectively (*A* and *B*). The numbers of total micronuclei (*closed circles*) and total nuclear buds (*open circles*) were determined in slides stained with DAPI (*C* and *D*). The numbers of DM+ micronuclei (*closed circles*) or DM+ nuclear buds (*open circles*) were determined in the slides hybridized with the purified micronuclei probe (*E* and *F*). These values are expressed as the frequency relative to the number of interphase nuclei scored (more than 1,000 for each point). The degree of synchronization was further evaluated by pulse labeling the cells for 30 min with BrdU. At the times indicated, the cells were harvested and the incorporated BrdU was detected by anti-BrdU antibody and rhodamin-conjugated secondary antibody, and then examined by confocal microscopy. The distribution of BrdU labeling inside each nucleus was classified into 6 patterns (*G*); i.e., *Pattern 0*, no labeling; *1*, only at the internal euchromatin; *2*, spread of the labeling to periphery; *3*, onset of labeling of peripheral heterochromatin; *4*, almost exclusive labeling of peripheral heterochromatin; *5*, labeling of peripheral and internal heterochromatin; and *6*, exclusive labeling of internal heterochromatin. At each time point after release from aphidicolin block, the frequencies of each pattern scored from 100 nuclei were plotted (*H*).

early S phase (Fig. 2 *G*, *Pattern 1*). The small but significant fraction of cells in early S phase most likely reflects the leakiness of the synchronization procedure. After the release from the aphidicolin block, these patterns progressed sequentially as shown in Fig. 2, *G* and *H*. At the peak of [^{3}H]thymidine uptake (4 h after release), 8.3% of cells still did not incorporate BrdU, suggesting that some cells may have been arrested irreversibly by aphidicolin.

The frequencies of micronuclei and buds were ascertained using the DNA specific dye DAPI (Fig. 2, *C* and *D*). We determined whether these structures contain amplified sequences by hybridizing all samples with the DM-painting probe obtained from micronuclei. The frequency of nuclei with buds at the G1/S boundary (i.e., $t = 0$) was nearly zero, increased dramatically as the cells progressed through early S phase ($t = 0$–5 h), declined in later S phase, and then gradually increased as the cells entered and progressed through a second S phase (Fig. 2 *C*). FISH analysis demonstrated that the buds enriched in DM sequences (DM + buds) showed the same time course (Fig. 2 *E*). The frequency of buds peaked concurrent with a BrdU labeling pattern characteristic of the onset of replication of peripheral heterochromatin (Fig. 2, *G* and *H*, *Patterns 3*). Importantly, the number of total micronuclei, or those with DMs, increased and declined in register with the number of nuclear buds (Fig. 2 *C*), suggesting that buds are precursors of micronuclei. We recently found that micronuclei containing DM sequences are released into the growth medium. The extracellular micronuclei were distinct from apoptotic bodies since they did not have condensed chromatin, the DNA was not degraded extensively, and the nuclear lamina were still intact (Shimizu, N., and G.M. Wahl, manuscript in preparation). Therefore, it is reasonable to propose that the decrease of micronuclei in late S phase reflects their release from the cells.

Whereas the frequency of nuclei with buds was low at the G1/S boundary, micronuclei were readily apparent (Fig. 2 *C*). One likely possibility is that these micronuclei were generated during the telophase of a previous cell cycle. We modified the synchronization strategy to include nocodazole to block cells in prometaphase after release from the aphidicolin block. This protocol restricts the analysis to micronuclei and buds generated within a single S phase, and to prevent buds or micronuclei produced from cells arrested before prometaphase of the previous cycle from entering the analysis (Cassimeris and Salmon, 1991). The treated cells entered and progressed through S phase at approximately the same rate as those not exposed to nocodazole (Fig. 2, compare *A* and *B*), and the mitotic index increased significantly by 8 h after release. The effectiveness of nocodazole treatment is also indicated by the inability of the drug-treated cells to progress into a second S phase over the time course used. Importantly, the number of nuclei with buds was again nearly zero at the G1/S boundary, and both the number of buds and micronuclei increased after release into nocodazole-containing medium. These data are consistent with the interpretation that nuclear buds and micronuclei can arise de novo during S-phase progression.

The kinetic and nuclear morphologic analyses of budding and micronucleation indicate that both events can occur during S phase. Since slowing replication fork progression may lead to DNA breakage (Eki et al., 1987; Linke et al., 1996), and micronuclei preferentially capture acentric fragments (Von Hoff et al., 1992; Shimizu et al., 1996), we determined whether replication inhibitors increase S phase micronucleation efficiency. The drugs tested included inhibitors of ribonucleotide reductase (HU, deferoxamine,

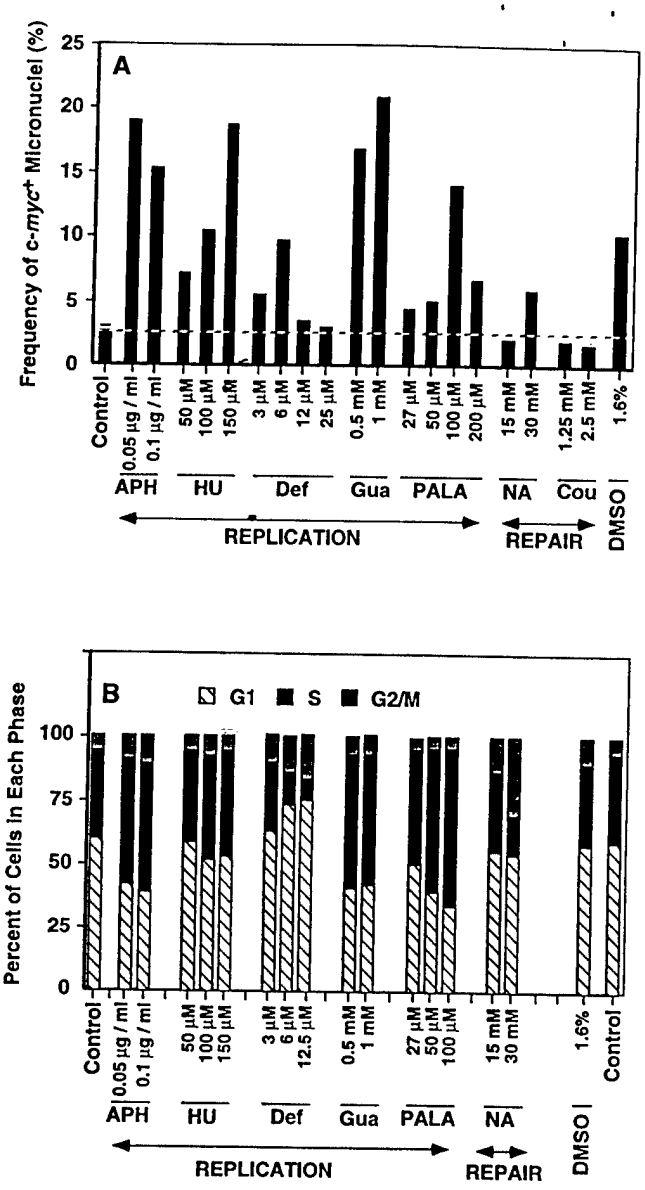

Figure 3. Effects of various drugs on induction of micronuclei and cell cycle distribution. COLO 320DM cells were treated for 3 d with inhibitors of DNA replication (*APH*, aphidicolin; *Def*, deferoxamine; *Gua*, guanazole; *HU*, hydroxyurea; and *PALA*), repair (*Cou*, coumarin; *NA*, nicotinamide) or the membrane-active agent DMSO at the concentrations indicated. (*A*) The treated cells were fixed with methanol/acetic acid and then hybridized with c-*myc* cosmid probe. The numbers of micronuclei that were stained brightly with c-*myc* probe were scored and expressed as *frequency of c-myc*$^+$ micronuclei (%) relative to the number of interphase nuclei scored (more than 1,000 for each point). (*B*) The cells were pulse labeled with BrdU (for 30 min) at the end of the drug treatment and then analyzed by flow cytometry as described under Materials and Methods to determine the fraction of cells in G1, S, and G2/M.

and guanazole), an inhibitor (PALA) of the carbamyl phosphate synthatase, dihydro-orotase, aspartate transcarbamylase (CAD) enzyme complex that catalyzes the first three steps of de novo pyrimidine biosynthesis, and aphidicolin. DNA synthesis inhibitors produced substantial increases in micronucleation, and this generally correlated with an increased fraction of cells in S phase (Fig. 3, *A* and *B*). A sharp decrease in micronucleation efficiency was observed for deferoxamine and PALA when these drugs were used at concentrations that severely inhibited S phase (Fig. 3 *B* and data not shown). These data indicate that micronucleation can result from inhibitors that retard replication fork progression and lengthen S phase.

We analyzed the effects of inhibitors that do not affect DNA synthesis to ascertain whether micronucleation can result from interfering with other DNA transactions such as DNA repair, or by interfering with membrane structure. Two inhibitors of poly(ADP-ribose) polymerase (nicotinamide and coumarin) were tested since ADP ribosylation has been implicated in DNA repair (Satoh and Lindahl, 1992), and inhibiting repair could increase the probability of generating acentric chromosome fragments. We tested the effects of DMSO, a membrane-active polar compound previously reported to reduce DM copy number in some tumor cell lines (Shima et al., 1989; Eckhardt et al., 1994). DMSO increased micronucleation without lengthening S phase (Fig. 3, *A* and *B*). Coumarin had no effect on micronucleation, whereas nicotinamide produced a small increase under conditions that apparently increased the amount of damage in the cells since there was a significant increase in the G2 fraction (Fig. 3, *A* and *B*). These data are consistent with the view that micronucleation efficiency can be increased by at least two mechanisms, one of which presumably involves perturbing replication fork progression, and another of which may involve events occurring outside of S phase.

Peripheral Nuclear Localization of DMs Correlates with Their Elimination by Budding

Insight into the mechanisms underlying the selective inclusion of DMs into nuclear buds and the formation of these structures in interphase was obtained by confocal microscopy. PFA fixation of nuclei was used for optimal preservation of nuclear morphology (Manuelidis and Borden, 1988).

Confocal sections from three representative nuclei isolated from rapidly growing untreated COLO 320DM cells are shown in Fig. 4, *A–C*. FISH revealed preferential localization of most DM sequences to the nuclear periphery, as indicated by the significant hybridization intensity, clustering, and number of DM signals at the extreme edge of each nucleus. Note the substantial deviation from a random distribution of DM sequences at the nuclear periphery (quantified in Fig. 5 *A* by measuring DM positions relative to the center of each nucleus in 100 interphase nuclei). By contrast, sequences amplified within chromosomes in the closely related cell line COLO 320HSR

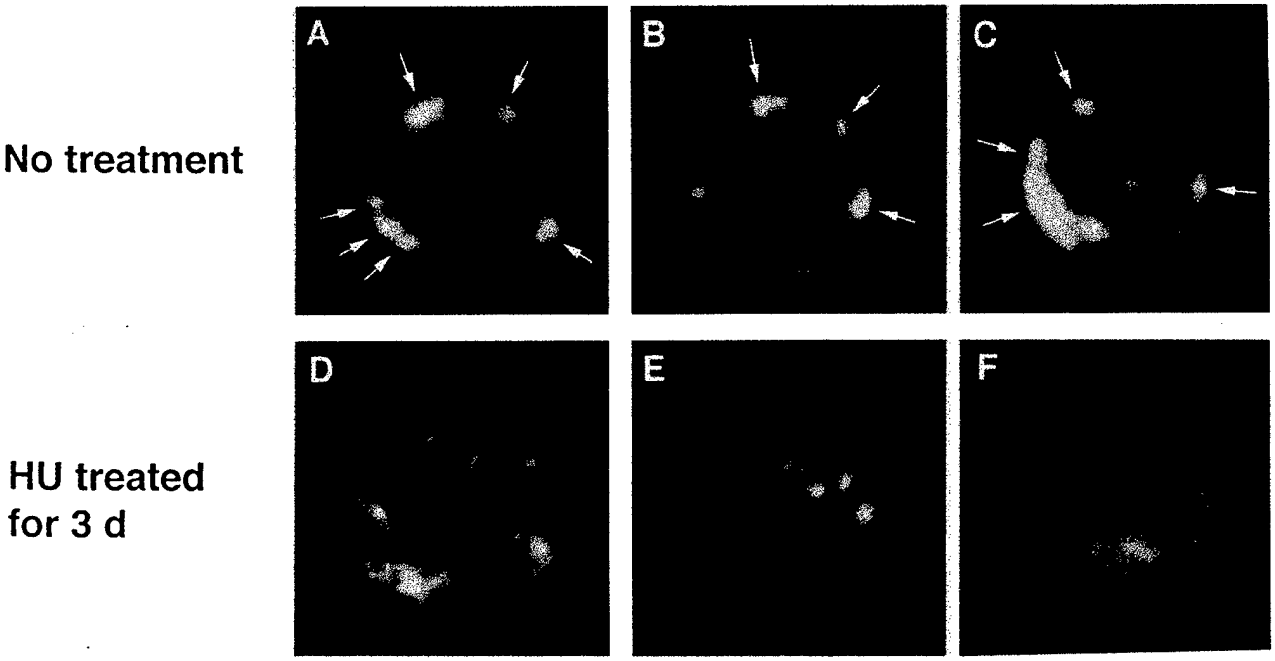

Figure 4. Localization of DMs in interphase COLO 320DM nuclei. PFA-fixed isolated nuclei from a culture of COLO 320DM cells were hybridized with a DM painting probe and then counterstained with PI. Optical sections near the center of each nucleus were obtained using confocal laser scanning microscopy. Each of three representative images of nuclei from the rapidly growing culture (*A–C*) and from a culture treated with 100 μM HU for 3 d (*D–F*) are shown. In untreated cultures, DMs preferentially located just beneath the nuclear membrane as indicated by the *arrows*. Very few peripheral DMs were detected in the nuclei from the HU-treated culture, and most of the signals localized well within the nucleus as shown.

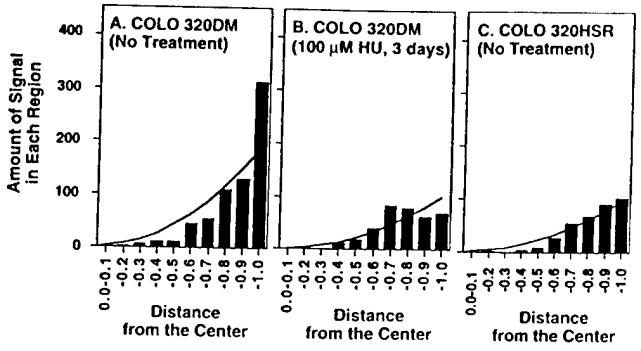

Figure 5. Quantitative analysis of the nuclear positions of DMs and chromosomally amplified sequences. The nuclei from a rapidly growing culture of COLO 320DM cells (*A*), COLO 320DM treated with 100 μM HU for 3 d (*B*), and rapidly growing COLO 320 cells with chromosomally amplified c-*myc* sequences (COLO 320HSR) (*C*) were hybridized with a probe for the c-*myc* amplicon. Sections intercepting the center of each nucleus were obtained as in Fig. 4. For each section, the position and intensity of each hybridized signal was measured. Each analysis represents measurements on 100 randomly chosen nuclei. The abscissa depicts the fractional distance from the nuclear center (*0*, center; *1*, periphery), and the ordinate is the number of signals detected at each position in 100 nuclei. The theoretical random distribution curves based on signals per nuclear vol at each position are shown in each graph as explained in greater detail in Materials and Methods.

showed a nearly random distribution throughout the nucleus (Fig. 5 *C*). HU treatment preferentially depleted DMs from the nuclear periphery (Fig. 4, *D–F* and Fig. 5 *B*, quantification) and then reduced the DM content per cell by approximately threefold as determined by competitive PCR amplification (for method see Shimizu et al., 1996; data not shown). Taken together with the data reported above, these results indicate that DM sequences located at the nuclear periphery are preferentially incorporated into nuclear buds, which are then removed from the nucleus through the formation of micronuclei.

Incorporation of Replicating DM Sequences into Nuclear Buds

The correlations between S phase progression, nuclear budding, and micronucleation reported above led us to investigate whether DM sequences undergoing replication are targeted for inclusion into buds. This possibility was examined by pulse labeling COLO 320DM cells with BrdU, and then hybridizing the isolated nuclei with the DM–FISH probe. Subsequent reaction with an anti-BrdU antibody and fluorescein-labeled secondary antibody enabled simultaneous detection of nuclei, buds, and micronuclei containing DMs that were undergoing DNA replication during the brief labeling interval. FISH analysis of two representative confocal sections (Fig. 6, *A–A″* and *B–B″*) shows that nuclear buds in these cells (*arrows*) contain highly concentrated DM sequences. The nuclei, nuclear buds, and peripheral regions of each nucleus incorporated BrdU, indicating that these buds were formed in nuclei that were synthesizing DNA at the time of bud formation. Table I shows that BrdU+, DM+ buds (type *1*, *1′*) represent 48% of the total population of DM-containing buds. Some nuclei incorporated BrdU, but the buds they produced were not labeled (type *2*, *2′*; 35%). We infer that some of these buds were also generated during S phase (as opposed to during the previous cycle), and that they did not reveal BrdU incorporation because their DNA was not undergoing replication during the brief BrdU incubation period. Samples in which neither nuclei nor buds labeled with BrdU may represent examples where buds were generated outside of S phase (type *3*; 17%). Alternatively, the presence of such buds (type *3*) may indicate that these structures persist after DNA replication has finished, and may be sources of some of the micronuclei observed to form during mitosis. These data reveal a strong correlation between DMs undergoing replication and their inclusion in buds and micronuclei, and they lead to a conservative estimate of ≥50% of the micronuclei produced during each cell cycle being generated during S phase.

The S phase micronucleation process described here superficially resembles the induction of "nuclear anomalies" by an apoptotic mechanism following γ irradiation (Duncan et al., 1985) or colchicine treatment (Duncan et al., 1984). However, the absence of highly condensed DNA in the nuclei-producing buds suggests that they were not undergoing apoptosis at the time of bud formation. To determine whether budding and micronucleation are separable from apoptosis, we determined whether buds contain the condensed, fragmented DNA that typifies apoptotic cells. Fragmented DNA was detected using the TUNEL assay in which terminal transferase is used to add BrdU to the 3′-OH groups generated by apoptotic DNA fragmentation (Gavrieli et al., 1992). The cells were also stained with PI to visualize all nuclei and buds. An example of the data obtained from such an analysis of COLO 320DM cells is shown in Fig. 6, *C–C″*. The TUNEL assay shows one cell with a lobular nucleus that exhibits a strong TUNEL reaction and typifies the fragmented, condensed DNA observed in apoptotic nuclei (Cohen, 1993). The PI staining in the middle panel reveals a cell producing a nuclear bud that does not stain by the TUNEL assay and does not exhibit the pycnotic structure of the apoptotic nucleus. Synchronization experiments showed that whereas ~5% of cells generated buds at the peak of S phase (e.g., refer to Fig. 2 *C*), only 0.5–1% were TUNEL positive. Other data (see Discussion) provide additional evidence that S phase budding and micronucleation do not require activation of an apoptotic program.

Micronucleation Occurs Infrequently in Normal Cells, and Is Increased upon p53 Inactivation

Micronucleation occurs at significantly lower rates in normal cells than tumor cell lines (Roser et al., 1989; Bondy et al., 1993). Since micronucleation can be induced by chromosome breakage (Heddle and Carrano, 1977), the observed increase in micronucleation in tumor cells might result from mutations that increase the probability of DNA breakage. The tumor suppressor p53 controls G1 arrest responses activated by DNA breakage and rNTP depletion induced by PALA treatment, and DNA breakage can occur in p53-deficient cell lines that enter S phase during PALA treatment (Livingstone et al., 1992; Yin et al., 1992; Linke et al., 1996). As reported above, PALA also induces

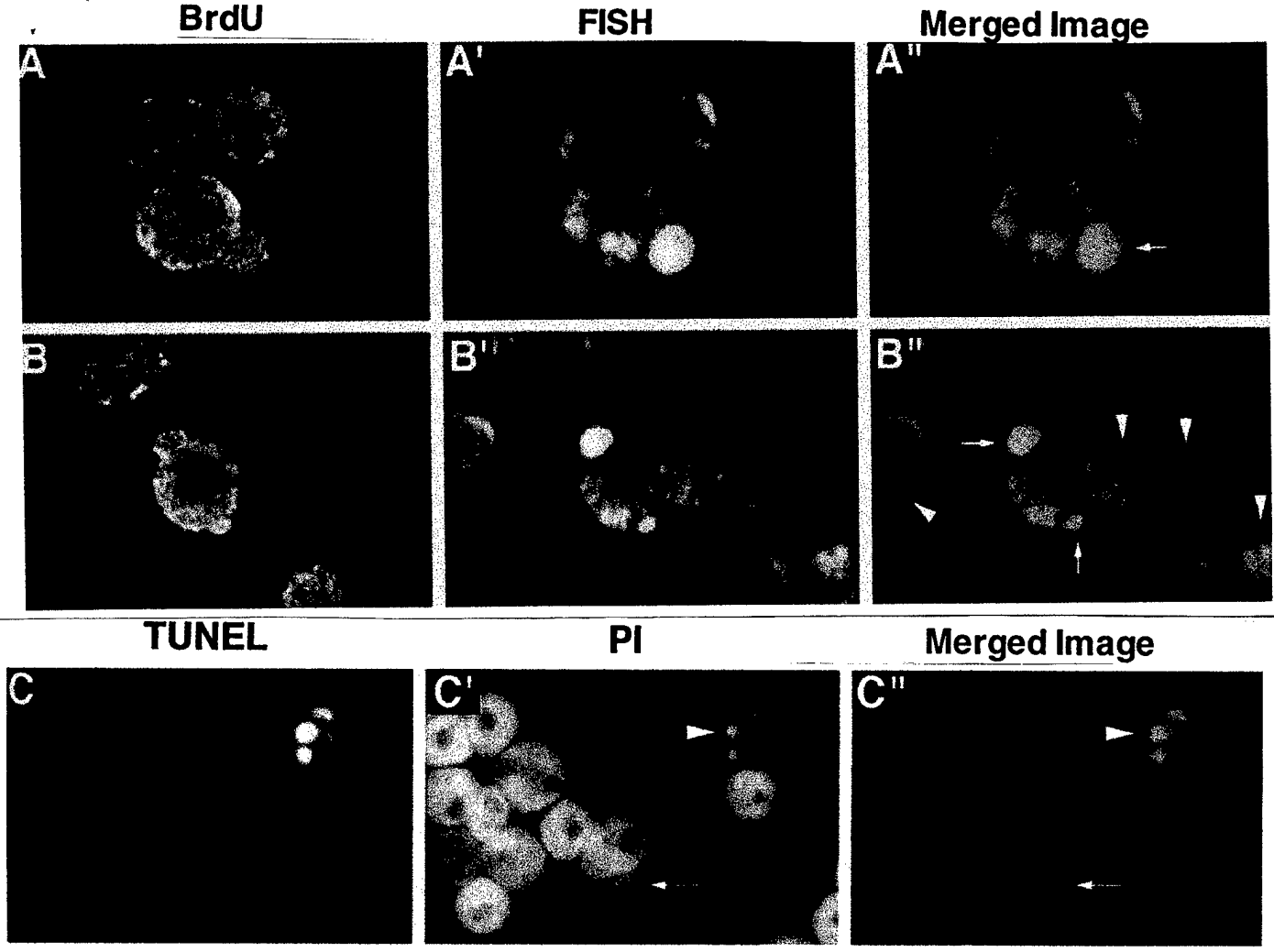

Figure 6. Analyses of DM–DNA replication and apoptosis in COLO 320DM cells. (*A* and *B*) A culture of rapidly growing COLO 320DM cells was pulse labeled with 10 μM BrdU for 1 h. The nuclei were isolated, fixed with PFA, hybridized with the biotinylated DM painting probe, and then detected using FITC-conjugated streptavidin as in Fig. 4. Sites at which BrdU was incorporated were detected with an anti-BrdU mouse monoclonal antibody followed by rhodamine-conjugated anti–mouse immunoglobulin. The double-labeled nuclei were examined using a confocal laser scanning microscope. The images of BrdU, FISH, and the merged images (*red*, *BrdU*; *green*, FISH) are shown for two representative fields. Nuclear buds that selectively entrap DMs are indicated by *arrows*, and the cells that were not in S phase during the pulse label (BrdU−) are indicated by *arrowheads*. (*C*) Analysis of apoptosis in COLO 320DM cells was done using the TUNEL method as described in Materials and Methods. This representative photograph is from one experiment in which COLO 320DM cells were treated with 100 μM HU for 3 d. The *arrow* points to a bud in a cell that is not undergoing apoptosis, whereas the *arrowhead* points to an apoptotic cell in the same field.

S-phase micronucleation in COLO 320 cells. These data led us to investigate whether p53 inactivation in normal diploid fibroblasts results in increased S-phase budding and micronucleation.

Human WS1 normal diploid fibroblasts, and two nearly isogenic derivatives generated by retroviral transduction of the neomycin phosphotransferase gene (WS1-neo) or an oncogenic derivative of the human papilloma virus E6 gene (WS1-E6) were used. The E6 gene product facilitates p53 degradation by a ubiquitin dependent pathway (Scheffner et al., 1990; Crook et al., 1991). Cell cycle checkpoint controls that regulate entry into S phase in the presence of DNA damage or limiting rNTP concentrations appear to be inactivated to equivalent degrees in human cells expressing mutant p53, oncogenic E6 protein, and mouse embryo fibroblasts with homozygous p53 knock out (Kastan et al., 1992; Kuerbitz et al., 1992; Livingstone et al., 1992; Yin et al., 1992; White et al., 1994; Linke et al., 1996; Linke et al., 1997). Importantly, we previously showed that the frequency of γ radiation induced micronucleation is higher in p53−/− MEFs than in wild-type MEFs (Huang et al., 1996). It is likely, therefore, that effects on micronucleation observed upon expression of oncogenic E6 protein relate to inactivation of p53 rather than to other proteins that may be affected by E6.

The data shown in Fig. 7 demonstrate that E6 gene expression increases the micronucleation rate of WS1 cells. WS1 cells exhibit a low micronucleation rate that is not increased by HU or PALA (Fig. 7 *A*). Consistent with our previous studies (Linke et al., 1996), PALA induced a G1 cell cycle arrest, whereas HU did not significantly affect the percentage of WS1 cells in S phase at the concentra-

Table I. Quantification of Budding and Micronucleation

Type	BrdU labeling in: DM+buds	BrdU labeling in: Nuclei	Frequency of DM+buds that belong to each type
1	+	+	26/60 (43%)
1′	±	+	3/60 (5%)
2	−	+	9/60 (15%)
2′	−	±	12/60 (20%)
3	−	−	10/60 (17%)

Nuclei from COLO 320DM were pulse labeled with BrdU and then analyzed for BrdU incorporation and DMs as described in Fig. 6. Nuclei were observed using epifluorescence microscopy. 60 nuclei with DM+buds were classified according to whether they labeled with BrdU (classes *1*, *1′*), whether the nuclei to which they were attached labeled with BrdU (classes 2, 2′), or whether neither bud nor nucleus labeled with BrdU (class *3*). The number of nuclei belonging to each type were scored and expressed as nuclei in that class/total number scored.

tion used (Fig. 7 *B*). By contrast, E6 expression increased the micronucleation efficiency of these fibroblasts growing under normal conditions, and both HU and PALA produced a substantial further increase in micronucleation rate (Fig. 7 *A*), which correlated with a significant increase in the number of cells in S phase (Fig. 7 *B*). The importance of an E6 target, which we infer to be p53, in limiting micronucleation is evident in other cell types since similar results were obtained using RPE-h and their E6 expressing derivatives (data not shown).

The elongation of S phase and induction of micronuclei by HU and PALA in both COLO 320DM and WS1-E6 cells led us to assess whether budding in S phase is the predominant mechanism of micronucleation in WS1-E6 cells. WS1-neo and WS1-E6 cells were arrested in G0 by serum deprivation and then released in the presence of aphidicolin to arrest the cells at the G1/S boundary (Fig. 7 *C*). Synchronization by serum depletion did not increase micronucleation rate (data not shown) and then the micronucleation and budding frequencies did not increase in S phase in WS1-neo cells (Fig. 7 *D*). By contrast, removal of aphidicolin from WS1-E6 cells resulted in significant increases in the frequencies of both nuclear budding and micronucleation as the cells progressed through S phase (Fig. 7 *D*). Since DNA damage does not induce apoptosis in either WS1 or WS1-E6 cells (Di Leonardo et al., 1994; Linke et al., 1996; Linke et al., 1997), the increased S-phase micronucleation observed in these cells occurs independent of an apoptotic program.

Discussion

Loss of cell cycle checkpoints during cancer progression creates a permissive environment for the initiation and propagation of chromosomal rearrangements such as amplification of cellular protooncogenes. The persistence or elimination of these structures within the nucleus can, respectively, promote or inhibit cancer progression. Interestingly, in human tumors analyzed at biopsy, oncogene amplification occurs most frequently in acentric chromosomal fragments such as DMs (Benner et al., 1991), and micronucleation represents a major pathway for the elimination of such structures (Von Hoff et al., 1992; Shimizu et al., 1996). Before this report, micronucleation had been considered to result from imperfect segregation of acentric chromosomal fragments or fragments of overly long chromosomes during karyokinesis (Heddle and Carrano, 1977; Heddle et al., 1983). The results presented here, by contrast, reveal that acentric DMs are sorted to the nuclear periphery during S phase, and are then selectively eliminated from the nucleus by micronucleation in advance of karyokinesis.

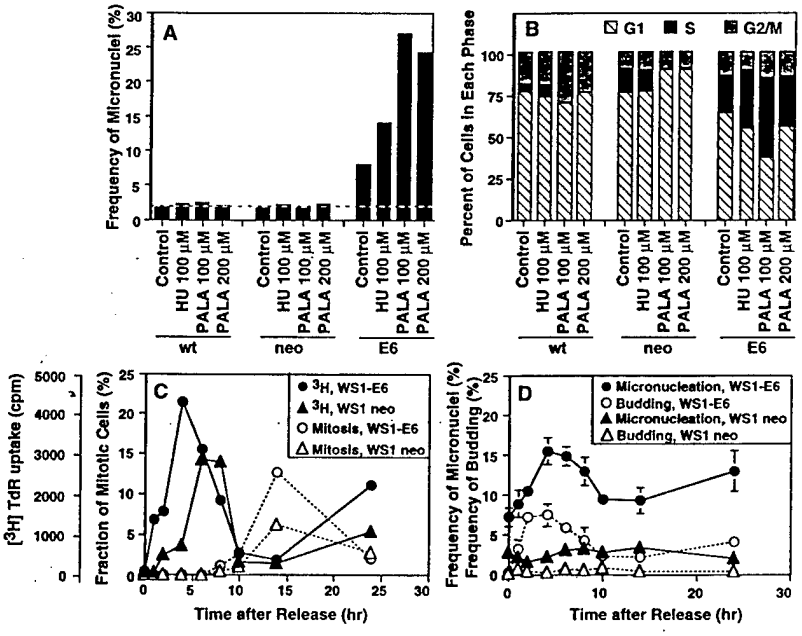

Figure 7. p53 deficiency increases S-phase budding and micronucleation in normal human diploid fibroblasts. Wild-type (*wt*) WS1 human diploid fibroblasts, or transformants expressing the neomycin resistance gene (*neo*) or both neo and the human papilloma virus E6 protein (*E6*) were generated by retroviral transduction. These cells were cultured in the absence or the presence of HU or PALA at the indicated concentrations for 3 d. (*A*) Cells grown on coverslips and treated as above, fixed, and then stained by DAPI. The number of micronuclei were scored and expressed as the frequency of micronuclei (%) relative to the number of interphase nuclei scored (more than 1,000 for each point). (*B*) Cell cycle effects of these drugs were examined as in Fig. 2 *B*. (*C* and *D*) The culture of WS1-neo (*open* and *closed triangles*) or WS1-E6 (*open* and *closed circles*) were synchronized at the G1/S boundary by the procedure described in Materials and Methods, and were released into growth medium. (*C*) Progression through S or M phase was monitored by the incorporation of [^{3}H]thymidine (*closed symbols*) or the fraction of mitotic cells determined by microscopic detection of metaphase figures (*open symbols*). (*D*) The cells on the coverslips were fixed, stained with DAPI, and then the frequencies of micronuclei relative to the number of interphase nuclei were scored. >500 nuclei were counted for each point and the results are expressed as mean ± SD for WS1-neo and WS1-E6 cells (three independent determinations for each strain; error bars for WS1-neo data points were smaller than the sizes of the symbols). Nuclear budding frequencies were determined on the same slides as used to determine the micronucleation index (more than 1,000 nuclei for each point).

S Phase Budding and Micronucleation May Be the Predominant Mechanism for DM Elimination

We infer that S-phase micronucleation is a general characteristic of human cells with an aberrant p53 pathway since this process was observed in a human tumor cell line, and p53-deficient normal human fibroblasts and epithelial cells. The cell synchrony and release experiments provide direct evidence that budding begins as cells enter S phase. The experiment summarized in Table I shows that BrdU+ micronuclei account for almost 50% of the total micronuclei generated during a single S-phase. It is reasonable to infer that a fraction of the 35% of BrdU− micronuclei derived from BrdU+ nuclei did not incorporate the label because their DNA did not replicate during the brief BrdU pulse used. Such micronuclei would, therefore, also have originated during S phase, but we have no means of providing a precise estimate of this fraction. The available data do, however, demonstrate that S-phase micronucleation mediated by budding is at least as common as the classic postmitotic process, and may be the predominant mechanism for removing DMs from human cancer cells.

The parallel increases in budding and micronucleation frequencies as cells enter and proceed through S phase, and the absence of a significant temporal lag between the two, is consistent with a precursor–product relationship in which a micronucleus is produced shortly after a bud is generated. The decrease in budding and micronucleation during the latter part of S phase is also consistent with a tight linkage to the replication program, and suggests that once micronuclei are generated during S phase, some may be released from the cell, whereas others may fuse to nuclei or be degraded intracellularly. Among these possibilities, we have direct experimental evidence that some micronuclei may be expelled from the cell through the cytoplasmic membrane since we have observed extracellular micronuclei containing three membranes and amplified c-*myc* genes (Fig. 8; Shimizu, N., and G.M. Wahl, unpublished data). We are currently testing whether such micronuclei are bound by a cytoplasmic membrane, and whether they can fuse to the same or other cells in the population (Fig. 8).

The baseline frequency of micronuclei observed in cultures at the beginning of S phase in which budding was not yet evident suggests that some micronuclei were generated in a previous cycle and persisted for an extended time, which would be consistent with previous reports of long lived micronuclei (Heddle et al., 1983). It is conceivable, therefore, that there are two classes of micronuclei that differ from each other by their stability, perhaps because of structural distinctions deriving from their mechanisms of formation. Consistent with the inference of multiple types of micronuclei, we found that micronuclei can differ in their lamin and nuclear pore contents (Shimizu, N., and G. Wahl, unpublished observations). Experiments in progress are designed to elucidate whether such differences correlate with stability and alternative mechanisms of formation.

S-phase Budding and Micronucleation Do Not Require Previous Engagement of an Apoptotic Program, but Can Result in Apoptosis

Apoptosis can generate nuclear blebs (Dini et al., 1996)

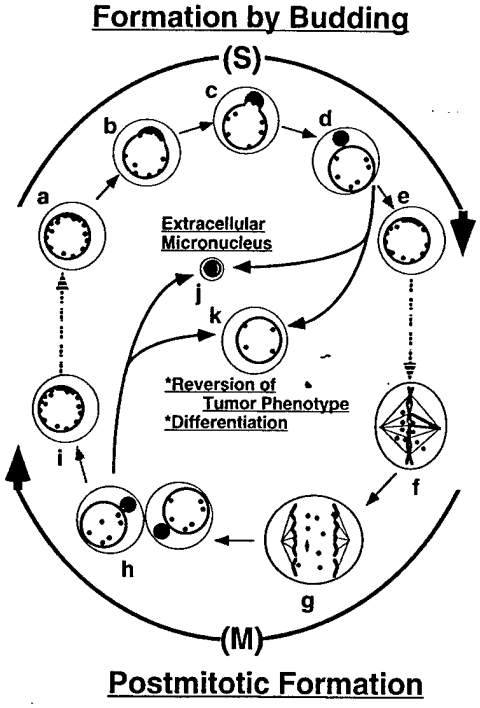

Figure 8. Model for the formation of micronuclei. Models for DM elimination by budding and micronucleation in S-phase and postmitotic micronucleation are shown. In the S-phase budding mechanism, DMs are preferentially located at the periphery of the interphase nucleus and then selectively encapsulated into nuclear buds that then pinch off to form micronuclei during DNA replication (*a–d*). This process is an alternative to the classical postmitotic mechanism of generating micronuclei depicted in *f–h*. One possible fate of these micronuclei is that they refuse to the main nucleus (*i* to *j* and *d* to *e*). At present, this is a speculation as there is no direct experimental evidence to support it. On the other hand, we do have direct evidence that micronuclei can be released into the culture supernatant as extracellular micronuclei, suggesting that they may be extruded through the cell membrane (*j*; Shimizu, N., and G.M. Wahl, unpublished data). Decrease in DM content can be achieved by either expulsion of micronuclei containing DMs from the cell, or by degradation of DM–DNA within intracellular micronuclei. In either case, loss of DM sequences from the nucleus results in reversion of the tumor phenotype, differentiation, or apoptosis (*k*; Von Hoff et al., 1992; Eckhardt et al., 1994; Shimizu et al., 1994).

and has been inferred to produce "nuclear anomalies" that resemble micronuclei (Duncan and Heddle, 1984; Duncan et al., 1985). However, our analyses show that nuclei that produced buds were not pycnotic and fragmented like apoptotic nuclei. Buds and micronuclei generated in COLO 320DM cells within a single S phase were not TUNEL positive, indicating that they did not contain fragmented DNA. Whereas apoptotic cells did arise in HU-treated COLO 320DM cultures, this required prolonged incubation, and occurred after a substantial fraction of the amplified c-*myc* genes had been removed. Furthermore, cells undergoing budding and micronucleation survived for several days, which is not expected if an apoptotic program were activated before micronucleation. A time course experiment similar to that described above (e.g., Fig. 2) revealed that budding increases during a single S phase whereas the fraction of cells undergoing apoptosis

remained roughly constant, and that most of those generating buds were not TUNEL positive (data not shown). Finally, though we have not observed apoptosis in normal fibroblasts (Di Leonardo et al., 1994), and loss of p53 function typically makes cells more resistant to apoptosis induced by growth conditions that can lead to DNA damage (White, 1994), S-phase budding and micronucleation were induced in normal diploid fibroblasts upon expression of oncogenic papillomavirus E6 protein, presumably due to elimination of p53 function. These observations lead us to propose that buds and micronuclei in COLO 320DM and WS1-E6 cells are produced by a mechanism that does not require prior engagement of the apoptotic program.

Mechanisms of Budding and Micronucleation

The molecular basis for inclusion of DMs in buds and micronuclei, and exclusion of similarly sized centric fragments from such structures (Shimizu et al., 1996), remains to be elucidated. We offer two potential explanations. The first relates to replication of DMs at an inappropriate nuclear location. Previous studies showed that heterochromatic DNA, such as that comprising inactive X chromosome–Barr bodies, typically replicates late in S phase at the nuclear periphery and is often enclosed within micronuclei, whereas the active X replicates earlier in S phase at a more internal position and is not subject to micronucleation (Dyer et al., 1989; Tucker et al., 1996). DMs are euchromatic and tend to replicate early in S phase, typically at approximately the same time as the native chromosomal locus (Carroll et al., 1991, 1993). However, as shown here, they can localize to and replicate at the nuclear periphery. Since chromosomes occupy specific territories within interphase nuclei (Cremer et al., 1993), the independence of DMs from chromosomes may prevent them from occupying the correct nuclear position and could explain their localization to the periphery. Peripheral localization may be a default position reflecting the inability of DMs to undergo the nuclear movements choreographed by centromeres and/or telomeres during S phase (DeBoni and Mintz, 1986; Ferguson and Ward, 1992; Vourc'h et al., 1993). Replication at such peripheral locations, or perhaps inability to move away from such sites into protected inner nuclear regions following replication, may then precipitate bud formation. A second explanation is that some chromosomal sequences may bind proteins that target the protein and associated nucleic acid to a particular nuclear location, such as the periphery. In the absence of a centromere/telomere, this protein–nucleic acid complex may be destined to form a nuclear bud until membrane fusion produces a micronucleus. This view is supported by recent studies in Tetrahymena demonstrating that *cis*-acting heterochromatic regions bound by the chromodomain protein Pdd1p are targeted to the nuclear periphery when the DNA is fragmented during macronuclear development (Madireddi et al., 1996). The peripheral, Pdd1p-associated acentric chromosomal fragments are then removed from the nucleus via micronucleation. The striking similarities between the observations in Tetrahymena and those reported here raise the intriguing possibility of an evolutionarity conserved process that distinguishes intact chromosomes from chromosome fragments or other acentric DNA such as DNA viruses to facilitate removal of the latter from the nucleus.

S-Phase Micronucleation Suggests That Loss of p53 Function Affects the Probability of Chromosome Breakage

Treatment of normal fibroblasts with HU or PALA did not induce S-phase micronucleation, whereas identical treatment of isogenic p53–deficient normal fibroblasts increased S-phase micronucleation frequency significantly. These data, along with previous observations, lead us to propose that this micronucleation increase most likely reflects a higher probability of DNA breakage occurring in the p53-deficient cells when they attempt DNA replication under adverse conditions. In support of this idea, micronucleation is an indicator of chromosome breakage, and has long been used as an assay for clastogens (Heddle et al., 1991). Inhibition of replication fork progression induces chromosome breakage in bacteria, yeast, and mammals (Eki et al., 1987; Kuzminov, 1995; Michel et al., 1997). Furthermore, replication inhibitors including PALA, methotrexate, and aphidicolin can induce chromosome breakage through expression of fragile sites (Kuo et al., 1994; Coquelle et al., 1997). Although the relationship between fragile site induction and p53 function has not been tested, the cell lines used for such studies were competent for gene amplification and consequently should have had a defective p53 pathway (Livingstone et al., 1992; Yin et al., 1992). Consistent with a breakage-moderating function of p53, we previously reported that PALA generates chromosome damage in cells with defective, but not normal, p53 function (Linke et al., 1996). Taken together, the data lead us to propose that p53 minimizes the frequency at which structural chromosomal alterations are induced during exposure to suboptimal growth conditions by at least two mechanisms. First, as shown previously, p53 mediates a G1 arrest in response to low rNTP pools, which prevents cells from entering S phase with inadequate precursors for DNA replication (Linke et al., 1996). Second, the data presented here suggest that p53 minimizes S-phase DNA breakage in cells that attempt DNA replication during diverse metabolic challenges. The mechanisms underlying the proposed S-phase function of p53 are currently under investigation.

Micronucleation and Cancer Treatment

Drugs that enhance S-phase budding should prove valuable for chemotherapy as they affect a process (micronucleation) and cytogenetic aberration (DMs) that are restricted to cancer cells. However, their efficacy is limited by the extent to which DM removal prevents further cell growth, or enhances sensitivity to other therapeutic strategies. In COLO 320DM cells, induction of the S-phase budding mechanism can decrease the number of DMs sufficiently to reduce plating efficiency in soft agar and tumorigenicity in vivo (Von Hoff et al., 1992). Interestingly, COLO 320HSR cells, which have approximately the same number of c-*myc* genes amplified within chromosomes, did not exhibit such phenotypic changes upon HU treatment and did not decrease c-*myc* copy number, even though they produced ap-

proximately the same number of micronuclei as COLO 320DM cells (Von Hoff et al., 1992). The same HU treatment conditions also induced apoptosis more rapidly and in a higher fraction of COLO 320DM than COLO 320HSR cells (data not shown). Similarly, HU treatment of HL-60 DM, but not HL-60 HSR cells, reduced c-*myc* copy number, induced differentiation, and later, apoptosis (Eckhardt et al., 1994; Shimizu et al., 1994). These results suggest that reduced tumorigenicity may result from induction of an apoptotic program when a sufficient number of extrachromosomally amplified sequences encoding oncogenes are removed from the cell. The recognition of a mechanism for the segregation and elimination of amplified sequences and other acentric DNA macromolecules, and the recognition that some agents can enhance this process, present important opportunities to expand and refine chemotherapeutic strategies, and to gain insight into the *cis*-acting elements and *trans*-acting factors that determine nuclear DNA localization.

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Histone–GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells

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Background: The amplification of oncogenes in cancer cells is often mediated by paired acentric chromatin bodies called double minute chromosomes (DMs), which can accumulate to a high copy number because of their autonomous replication during the DNA synthesis phase of the cell cycle and their subsequent uneven distribution to daughter cells during mitosis. The mechanisms that control DM segregation have been difficult to investigate, however, as the direct visualization of DMs in living cells has been precluded because they are far smaller than normal chromosomes. We have visualized DMs by developing a highly sensitive method for observing chromosome dynamics in living cells.

Results: The human histone H2B gene was fused to the gene encoding the green fluorescent protein (GFP) of *Aequorea victoria* and transfected into human HeLa cells to generate a stable line constitutively expressing H2B–GFP. The H2B–GFP fusion protein was incorporated into nucleosomes without affecting cell cycle progression. Using confocal microscopy, H2B–GFP allowed high-resolution imaging of both mitotic chromosomes and interphase chromatin, and the latter revealed various chromatin condensation states in live cells. Using H2B–GFP, we could directly observe DMs in living cancer cells; DMs often clustered during anaphase, and could form chromosomal 'bridges' between segregating daughter chromosomes. Cytokinesis severed DM bridges, resulting in the uneven distribution of DMs to daughter cells.

Conclusions: The H2B–GFP system allows the high-resolution imaging of chromosomes, including DMs, without compromising nuclear and chromosomal structures and has revealed the distinctive clustering behavior of DMs in mitotic cells which contributes to their asymmetric distribution to daughter cells.

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Background

In eukaryotes, segregation of sister chromatids during mitosis requires spindle fiber attachment to the kinetochores formed at centromeric DNA. Cancer cells often harbor abnormal chromosomes such as dicentric or acentric chromosomes, however, which would be expected to behave anomalously. Double minute chromosomes (DMs) are paired chromatin bodies that have been reported in as many as 50% of human tumors but have never been observed in normal cells [1,2]. As DMs lack functional centromeres, they do not segregate by the same mechanism used by normal chromosomes. DMs can accumulate to a high copy number because of their autonomous replication during the DNA synthesis (S) phase of the cell cycle and their subsequent uneven distribution to daughter cells during mitosis. As DMs contain a diversity of amplified oncogenes [3], their uneven segregation and accumulation increases the malignant potential during tumor progression. On the other hand, as DM loss can decrease tumor cell viability [4], understanding the mechanism of DM segregation could lead to the identification of highly selective anti-neoplastic agents that specifically disrupt the transmission of DMs to daughter cells.

Observations of fixed chromosomes in DM-harboring cancer cells have provided some insights into DM segregation during mitosis [5,6]. Fixation and permeabilization of cells may cause artificial distortions of chromosome distribution, however, and could perturb intracellular structures. An ideal strategy for examining the dynamics of DM segregation would involve their direct visualization in living cells. DMs vary in size, however, and many are at the size limit of conventional cytogenetics (~1–2 × 10^6 bp) [2], which has prevented their detection in cycling cells. This report describes a fluorescent labeling system with sufficient sensitivity to visualize DMs *in vivo*, and that enables analyses of their segregation dynamics in real time during mitosis.

One approach to label chromosomes in living cells involves the fluorescent tagging of proteins that localize to chromosomes. The nucleosome is the fundamental repeating unit

of chromatin. Each nucleosome core particle consists of an octamer of core histones with 146 bp of micrococcal-nuclease-resistant DNA wrapped around it [7]. As histones are the principal structural proteins of eukaryotic chromosomes, they are attractive targets for fluorescent labeling. Purified calf thymus histones (H2A and H2B) conjugated with rhodamine have been microinjected into *Drosophila* embryos to analyze cell lineage relationships [8] and chromosomal condensation and decondensation events [9]. The success of this approach demonstrates the utility of fluorescently labeled histones to study chromosomal dynamics in living cells.

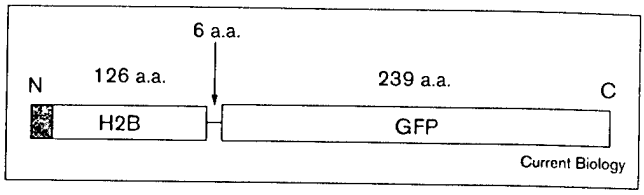
The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* retains its fluorescent properties when recombinant GFP proteins are expressed in eukaryotic cells [10]. GFP fusion proteins have been successfully targeted to specific subcellular organelles and structures including the nucleus, plasma membrane, mitochondrion, cytoskeleton, and Golgi apparatus [11–13]. Recently, GFP tagging also enabled visualization of specific chromosomal regions [14–16]. These results indicate the potential utility of a histone–GFP fusion protein to fluorescently label chromosomes in living cells. The feasibility of this approach is indicated by the observation that a fusion protein of GFP and yeast histone H2B localized properly in yeast nuclei [17]. Here, we show that a fusion protein of GFP and human H2B (H2B–GFP) is incorporated into nucleosome core particles without perturbing cell cycle progression. H2B–GFP bound chromosomes and DMs with high specificity, allowing them to be easily observed using a confocal microscope. We describe for the first time the behavior of DMs during mitosis in living cells. Our results reveal that DMs often cluster in anaphase cells and attach to groups of segregating chromosomes. Sometimes, segregating daughter chromosomes are connected by DM 'bridges' spanning the midplane of anaphase cells. Time-lapse observation revealed that cytokinesis severs the DM bridges, resulting in asymmetric distribution of DMs to the daughter cells.

Results

Stable expression of H2B–GFP in HeLa cells

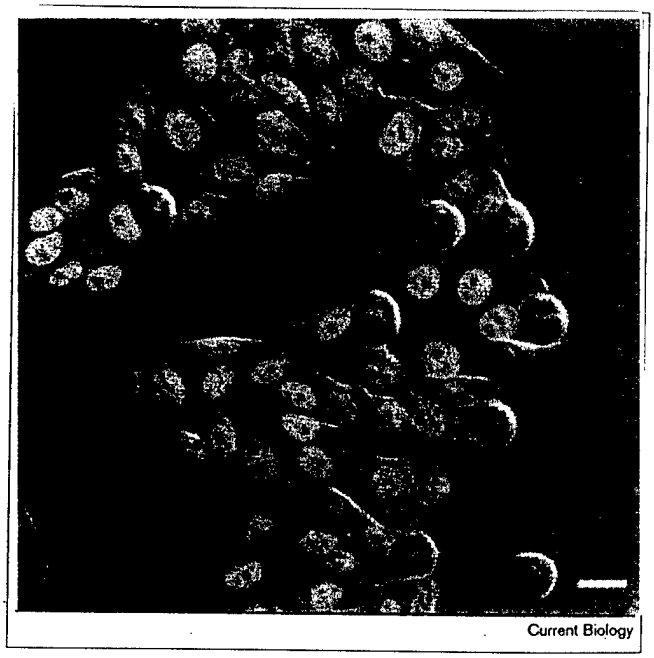
The cDNA encoding human H2B was tagged at its carboxyl terminus with DNA encoding codon-optimized enhanced GFP [18] (Figure 1), and the chimeric gene was subcloned into a mammalian expression vector. The construct was introduced into the human HeLa cell line by transient transfection, and fluorescence microscopic observation indicated that H2B–GFP protein localized to interphase nuclei and mitotic chromosomes (data not shown). To analyze the effects of constitutive H2B–GFP expression on cell cycle progression, we transfected HeLa cells and cultured them under drug selection (blasticidin) to obtain clones that stably expressed the H2B–GFP transgene. GFP-positive colonies arose in about 10% of blasticidin-resistant colonies, while other colonies (~90%) were negative for GFP for unknown reasons. We obtained several stable cell lines expressing H2B–GFP. A cell line with uniform, high-level expression of H2B–GFP was chosen for further analyses (Figure 2). The expression level of H2B–GFP in this cell line was stable for more than three months in the absence of continuous blasticidin selection. The high degree of stability of the integrated H2B–GFP gene in the absence of selection strongly suggests that chromosome stability is unimpaired by constitutive H2B–GFP expression. The mitotic index and the growth rate of this cell line were similar to those of the

Figure 1



The H2B–GFP chimeric protein. The H2B protein was tagged with GFP at its carboxyl terminus; the length, in amino acids (a.a.), of each region and of the junction between H2B and GFP is indicated. The amino (N) and carboxyl (C) termini of the fusion protein are indicated and the histone amino-terminal tail is shown as a gray box.

Figure 2



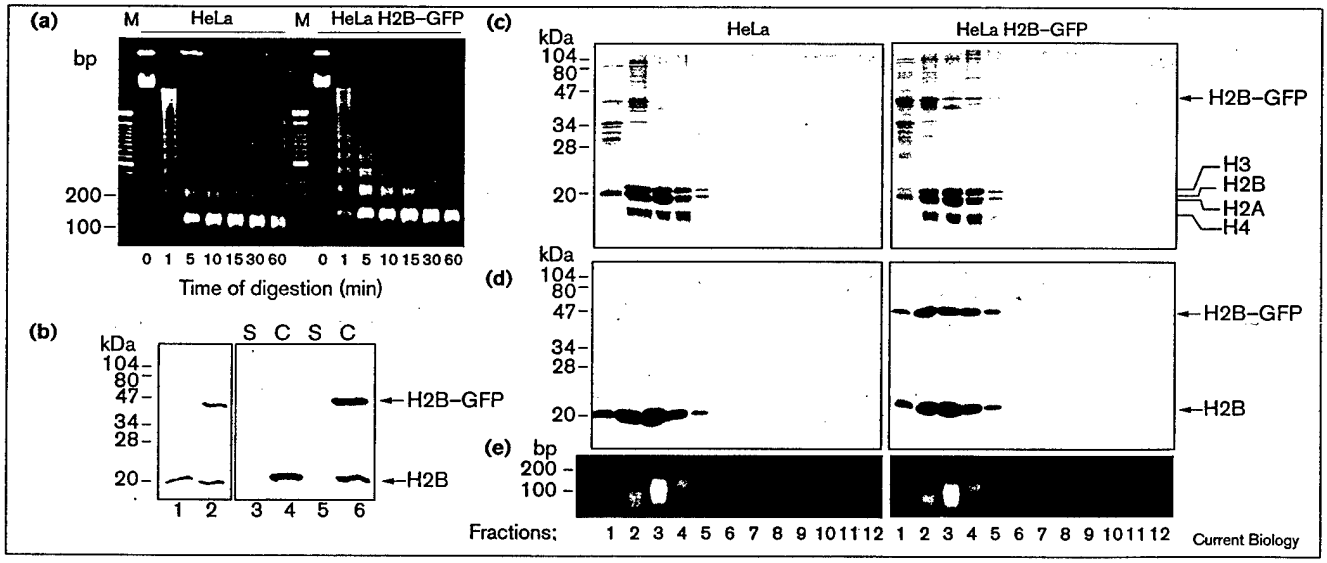
Cells expressing H2B–GFP. A confocal microscopic image of live HeLa cells constitutively expressing H2B–GFP; the GFP fluorescence (green) was overlaid onto a differential interference contrast image. The figure shows that H2B–GFP is detected highly efficiently in cells in all phases of the cell cycle and that H2B–GFP is contained solely in the nucleus. The scale bar is 25 μm.

parental HeLa cells (data not shown). We also used a cell line stably expressing a fusion protein of H2B fused at its amino terminus to GFP and got essentially the same results (data not shown).

H2B–GFP is incorporated into nucleosomes

We biochemically fractionated nucleosome core particles from cells expressing H2B–GFP and analyzed for the presence of the fusion protein to determine if it was a component of nucleosome core particles. Mononucleosomes were generated by extensive micrococcal nuclease digestion of the isolated nuclei expressing H2B–GFP (Figure 3a). Digested chromatin was pelleted by subsequent centrifugation of the nuclease-treated nuclei. The supernatant and pellet, together with whole cell lysate of HeLa cells and HeLa cells expressing H2B–GFP, were analyzed by western blotting using an anti-human-H2B antibody. The majority of the expressed H2B–GFP protein was recovered in the pelleted chromatin fraction, and little if any was detected in the supernatant (Figure 3b, compare lane 5 with lane 6). The chromatin fraction was further fractionated by sucrose gradient centrifugation in the presence of 0.5 M NaCl to dissociate histone H1 [19]. Electrophoretic analysis of the DNA showed that fractions 2 through 4 (predominantly fraction 3) contained DNA of about 146 bp (the size expected for the DNA wrapped around nucleosome core particles; Figure 3e). The proteins in the samples were analyzed by gel electrophoresis; H2B–GFP protein and core histones were identified in the mononucleosome fractions by Coomassie staining (Figure 3c). Aliquots of the same samples were analyzed by western blotting using anti-human-H2B antibody to specifically detect H2B and H2B–GFP protein (Figure 3d). The results demonstrate that the H2B–GFP fusion protein is in the mononucleosome fractions of the sucrose gradient and that its distribution parallels that of native histones in the gradient. The relative amounts of H2B–GFP and endogenous H2B in the purified mononucleosomes (Figure 3d) were comparable to their relative amounts in the whole cell lysate (Figure 3b, lane 2), suggesting that H2B–GFP protein is incorporated into nucleosomes efficiently. H2B–GFP association with the nucleosome core particle was stable under conditions that dissociate histone H1 [19], suggesting that adventitious aggregation of H2B–GFP protein with chromatin is unlikely (see Discussion).

Figure 3



H2B–GFP is incorporated into mononucleosomes. **(a)** Micrococcal nuclease digestion of nuclei from HeLa cells and HeLa cells expressing H2B–GFP. Isolated nuclei were digested for 0, 1, 5, 10, 15, 30, and 60 min, as indicated, and the DNA protected from digestion by the binding of nucleosomal core proteins was analyzed by 1.5% agarose gel electrophoresis. The markers used (M) were 100 bp ladders. **(b)** Whole cell lysate (25 μg each from HeLa cells, lane 1, or HeLa cells expressing H2B–GFP, lane 2), supernatants (S) and soluble chromatin fractions (C) of the digested nuclei (10 μg each from HeLa cells, lanes 3 and 4, or HeLa cells expressing H2B–GFP, lanes 5 and 6) were analyzed by western blotting using anti-human-H2B antibody. Soluble chromatin fractions were prepared as described in Materials and methods. H2B–GFP protein (approximately 45 kDa) and endogenous H2B protein are indicated. **(c)** Sucrose gradient analysis of mononucleosome populations. The mononucleosome protein–DNA complexes from HeLa cells and HeLa cells expressing H2B–GFP, prepared by micrococcal nuclease digestion, were purified through parallel 5–30% sucrose gradients. Proteins from each fraction were extracted and analyzed by electrophoresis through SDS–15% polyacrylamide gels and Coomassie staining. H2B–GFP protein and the native core histone proteins (H2A, H2B, H3 and H4) are indicated. **(d)** Aliquots of the fractions in (c) were electrophoresed and analyzed by western blotting using anti-human-H2B antibody. H2B–GFP protein and endogenous H2B protein are indicated. **(e)** DNA in each fraction was analyzed by 1.5% agarose gel electrophoresis.

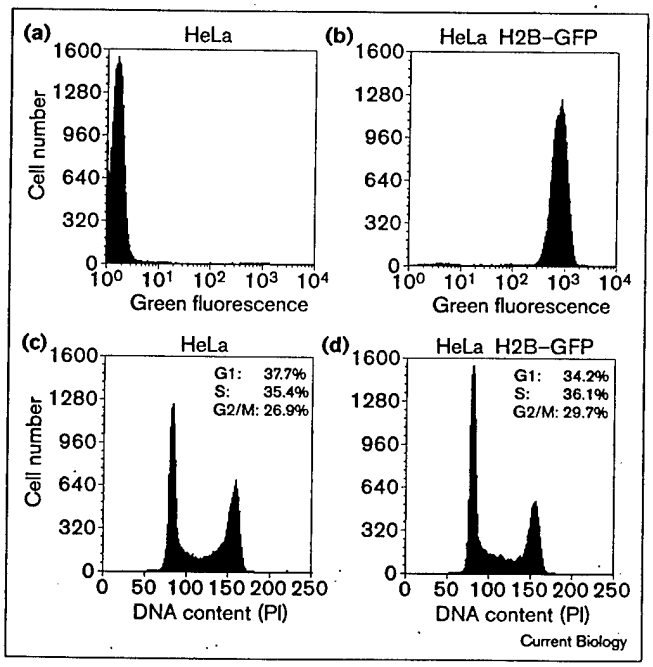
H2B–GFP incorporation does not inhibit cell cycle progression

It was conceivable that GFP tagging of H2B protein could affect chromatin structure and perturb cell cycle progression as a consequence. Therefore, the cell cycle distribution of the established cell line expressing H2B–GFP was analyzed to ascertain differences in cell cycle progression relative to the parental cell population. Asynchronous HeLa cells and the transformant expressing H2B–GFP were fixed with ethanol, stained with propidium iodide (PI), and analyzed by fluorescence-activated cell sorting (FACS). The green emission of GFP-labeled cells produced an approximately three-log shift from parental HeLa cells (Figure 4a,b). DNA content was determined by measuring the red emission of PI (Figure 4c,d). The results indicate that the cell cycle distribution of asynchronous HeLa cells expressing H2B–GFP is indistinguishable from that of the parental HeLa cells, clearly demonstrating that the H2B–GFP protein has little, if any, effect on cell cycle progression.

H2B–GFP decorates chromosomes in living cells

Cells expressing H2B–GFP were observed using confocal microscopy to determine the pattern of chromatin staining in interphase and mitosis. As shown in Figure 5, H2B–GFP enabled highly sensitive chromatin detection in all phases of the cell cycle. Fixation and permeabilization of the cells, which might cause artificial distortion of intracellular structure, was not required to obtain such images. H2B–GFP was highly specific for nuclear chromatin as no fluorescence was observed in the cytoplasm. In addition, H2B–GFP provided a remarkable level of sensitivity. For example, a chromatin structure that appeared to be a pair of lagging sister chromatids with a centromeric constriction was readily observed (Figure 5e). The fine intranuclear chromatin architecture in interphase nuclei visualized by H2B–GFP was consistent with the previously reported deconvoluted optical-sectioning images of fixed nuclei obtained by 4′,6′-diamidino-2-phenylindole (DAPI) staining [20]. Chromosome spreads of the H2B–GFP-expressing cells also showed that the GFP fluorescence patterns were identical to the patterns obtained using DAPI (Figure 5i,j).

Figure 4



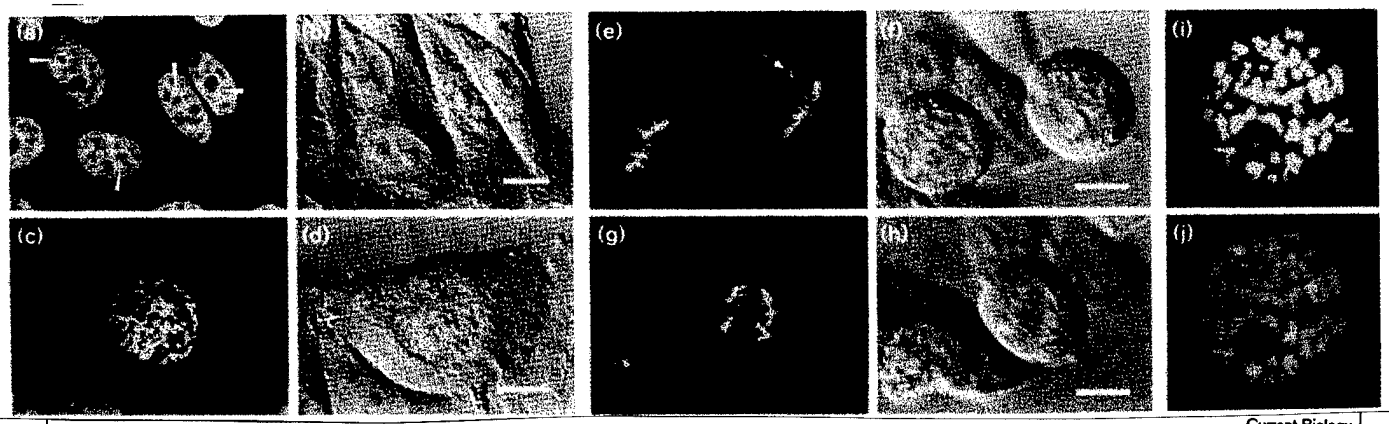
H2B–GFP expression does not affect cell cycle progression. **(a,c)** HeLa cells and **(b,d)** HeLa cells expressing H2B–GFP were fixed with ethanol, stained with PI, and analyzed by FACS. (a,b) Flow cytometry histograms of GFP fluorescence. (c,d) Flow cytometry histograms of the DNA content determined by PI staining. The estimated proportion of cells in the G1, S, and G2/M fractions are indicated.

We also observed perinucleolar regions densely stained with H2B–GFP that resembled chromocenters in interphase nuclei (Figure 5a). A previously described feature of chromocenters is that they are heterochromatic and often contain centromeres [21,22]. Double staining with centromere antibodies and H2B–GFP demonstrated that certain regions with intense H2B–GFP staining possessed multiple centromeres (Figure 6). From this result, coupled with the concordance of H2B–GFP staining and DAPI staining (Figure 5i,j), we conclude that H2B–GFP staining reflects the density of packing of DNA in different regions of the nucleus. Thus, chromosomal domains that have previously only been examined in fixed cells may be monitored using the H2B–GFP method in living cells.

Visualization of DMs in living cells

We applied our highly sensitive H2B–GFP chromatin-labeling technique to the analysis of DMs in living cells. A retroviral vector was constructed to enable the efficient transfer and expression of H2B–GFP in a broad range of host cells. We used a vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped retroviral vector to obtain high viral titers [23]. COLO320DM cells harboring DMs containing an amplified c-*myc* gene [24,25] were infected with the H2B–GFP retrovirus, and two days later over 90% of the cells expressed H2B–GFP protein. FACS analyses revealed that cell cycle progression of COLO320DM cells was not affected by H2B–GFP expression (data not shown). We collected serial-sectioning images of living COLO320DM cells expressing H2B–GFP using confocal microscopy. We noticed that small fluorescent dots were frequently observed in mitotic cells (Figure 7a,b). The sizes of these dots were 0.7–0.85 μm in diameter, corresponding to the size of DMs in this cell line. We found that they frequently associated in clusters in anaphase cells where they were attached to normal chromosomes (Figure 7a). Sometimes they aligned in regular arrays and

Figure 5

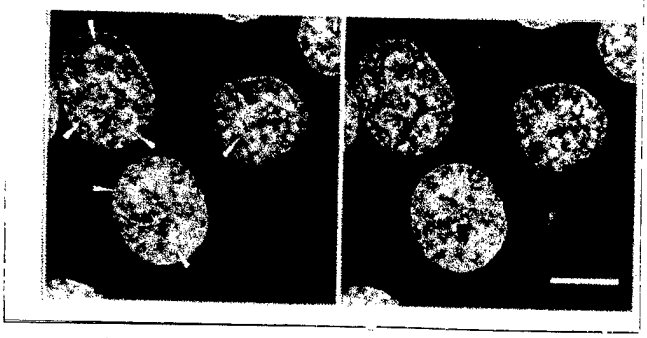


Localization of H2B–GFP protein. **(a–h)** Confocal microscopic images of live HeLa cells expressing H2B–GFP in various cell cycle phases. (a,c,e,g) The GFP fluorescence and (b,d,f,h) the corresponding differential interference contrast images are shown for (a,b) interphase, (c,d) prophase, (e,f) metaphase and (g,h) anaphase cells. Perinucleolar densely staining regions of H2B–GFP are indicated by arrowheads in (a). A pair of lagging sister chromatids with a centromeric constriction is indicated by an arrow in (e). The scale bars are 10 μm. **(i)** GFP localization and **(j)** DAPI staining of fixed chromosome spreads of HeLa cells expressing H2B–GFP.

formed an extended bridge between segregating groups of daughter chromosomes (Figure 7b).

In order to confirm that these dot-like chromatin bodies were DMs, mitotic COLO320DM cells were fixed with or without colcemid treatment, and the DM distribution was analyzed by fluorescence *in situ* hybridization (FISH) using a c-*myc* cosmid probe. Whereas chromosome spreads of colcemid-treated cells usually had dispersed DMs (Figure 7c), DMs were observed to form clusters in untreated mitotic cells (Figure 7d,e). The DMs detected using FISH were strikingly similar to the dot-like structures observed in H2B–GFP-expressing cells (compare Figure 7a,b to Figure 7d,e). The results strongly suggest that the dot-like chromatin bodies observed in living cells are DMs. We found that most of the observed mitotic cells contained clustered DMs, although the number of DMs varied from cell to cell. Approximately 30% of the anaphase cells showed bridge formations involving DMs. We therefore conclude that the clustering of DMs and their unbalanced distribution to daughter cells during mitosis are very common events in this cell line.

Figure 6



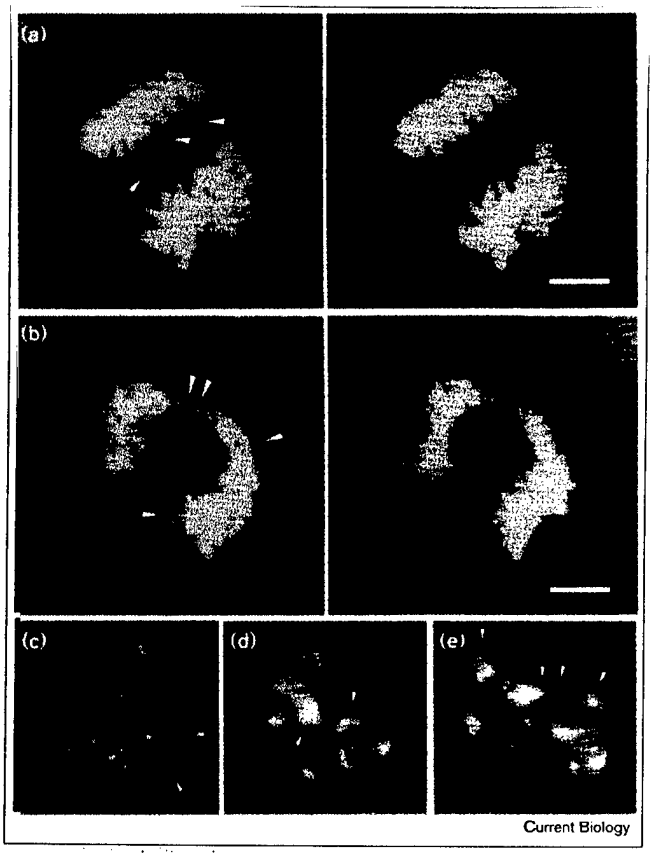
Perinucleolar regions densely stained with H2B–GFP possess multiple centromeres. Stereoscopic images of centromere localization in H2B–GFP-expressing HeLa cells. Centromeres were detected by immunofluorescence using a human anti-centromere antiserum. Localization of centromeres (red) and H2B–GFP (green) are indicated. Perinucleolar heterochromatic domains are indicated by arrowheads. The scale bar is 10 μm.

We next analyzed DM segregation by making time-lapse observations using an epifluorescence microscope (Figure 8). Clustered DMs were attached by the extended arms of normal segregating chromosomes, forming a chromosome bridge (Figure 8a). This bridge was further extended as daughter chromosomes segregated (Figure 8b). Subsequently, the bridge was severed by the process of cytokinesis (Figure 8c), and the cluster of DMs appeared to be unevenly distributed to daughter nuclei (Figure 8d). These results clearly demonstrate that DMs frequently cluster in anaphase cells, sometimes forming chromosomal bridges, and that their uneven distribution to daughter cells can result from cytokinesis severing DM bridges asymmetrically.

Discussion

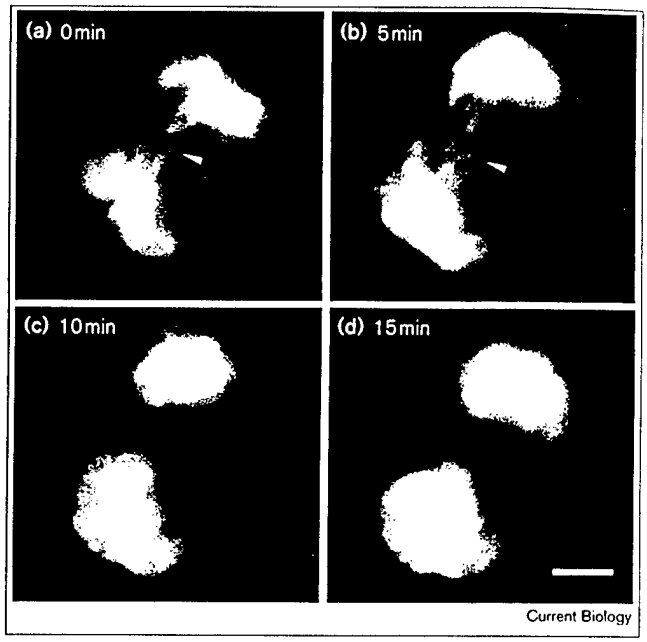
We have described a novel strategy to fluorescently label chromosomes in living cells and the successful application of this strategy to observe DMs in living cells. Despite the large size of the GFP tag (239 amino acids), it has been shown in numerous cases that GFP-tagged proteins are functional and localize properly [11–13]. Such is also the case with the histone H2B–GFP fusion protein. Our experimental data demonstrate the co-fractionation of

Figure 7



DMs cluster in anaphase cells. **(a,b)** Stereoscopic images of live COLO320DM cells expressing H2B–GFP. Clustered dot-like chromatin bodies (shown by arrowheads) together with segregating daughter chromosomes were visualized by GFP fluorescence (green). The scale bars are 5 μm. **(c)** DMs were detected in a metaphase spread of colcemid-treated COLO320DM cells by fluorescence *in situ* hybridization (FISH) using a biotinylated c-*myc* cosmid probe and fluoroscein-isothiocyanate–avidin. Chromosomes were counterstained with PI. **(d,e)** Asynchronously growing COLO320DM cells were directly fixed on chamber slides without colcemid treatment and processed for FISH analyses with the c-*myc* cosmid probe. Clustered DMs (arrowheads) and segregating daughter chromosomes (PI stained; red) are shown. Fluorescence images were collected using an epifluorescence microscope.

Figure 8



Transmission of DMs to daughter cells visualized in living cells. Time-lapse imaging of an H2B–GFP-labeled COLO320DM cell from late anaphase to telophase. A cluster of lagging DMs (shown by arrowheads) formed a bridge between segregating chromosomes, and the bridge was severed by cytokinesis. Fluorescence images were collected at the indicated times using an epifluorescence microscope equipped with a video camera. The scale bar is 5 μm.

H2B–GFP with mononucleosomes under high ionic strength (0.5 M NaCl). The primary interactions responsible for the stability of nucleosomes are electrostatic as nucleosomes can be dissociated into their DNA and histone components by elevating ionic strength. Histone H1, as well as non-histone proteins, dissociates from the nucleosomes at 0.5 M NaCl [7,19]. It is therefore likely that H2B–GFP protein is incorporated into nucleosome core particles rather than just affiliating with the core particles by a non-specific electrostatic interaction.

The strategy described in this paper offers significant advantages over other chromosome-labeling methods. Although fluorescent labeling of mammalian chromosomes in living cells has been demonstrated using Hoechst 33342 [26,27], each cell line must be analyzed individually to optimize the time of drug exposure and concentration of the drug [28]. Furthermore, as Hoechst 33342 is excited maximally near 350 nm and high intensities of ultraviolet (UV) irradiation can damage cells and produce cell cycle delay or arrest, the level of UV excitation must be controlled carefully. In addition, Hoechst 33342 affects cell cycle progression, arresting cells in G2 phase [29]. Intercalating DNA drugs, like dihydroethidium, may cause mutations in the DNA by interfering with DNA replication [28]. Microinjection of rhodamine-labeled histones, successfully used in *Drosophila* [8,9], is not suitable for analyzing a large population of mammalian cells. In contrast to these methods, the enhanced GFP [18] used in this study is excited with blue light (490 nm), which is less damaging than the UV-light excitation required for Hoechst. Moreover, constitutive expression of H2B–GFP from the integrated transgene enables long-term analyses without perturbing cell cycle progression (Figure 4). As the primary structures of histone proteins are well conserved among different species [30], it is likely that the H2B–GFP described here will be useful for cells of different species. This notion is supported by our ability to use the H2B–GFP vector in mouse, hamster, and monkey cells (data not shown).

The H2B–GFP strategy described in this report has a wide variety of applications for studying chromosome dynamics. For example, it can be used as a transfection marker that readily enables one to identify mitotic cells using fluorescence microscopy. H2B–GFP fluorescence persists in cells fixed in ethanol (Figure 4), which is useful for FACS analyses. As the intensity of H2B–GFP fluorescence depends on the chromosome condensation states in interphase cells (Figure 6), one can study chromosome condensation and decondensation in live cells [9]. The method may be especially useful for real-time analysis of apoptosis by enabling visualization of chromatin fragmentation and hypercondensation in living cells, as well as for studies of the effects of oncogenes on chromosome stability during tumor progression [31].

Using a retroviral vector expressing H2B–GFP, we have demonstrated that both normal chromosomes and small DMs can be readily distinguished in live cells. H2B–GFP enabled the observation of chromosomes in their native state without the need for fixation and permeabilization procedures, which can cause artificial distortion of intracellular structures. Our observations validate and extend a previous report in which DMs in fixed mitotic cells were observed to associate with the condensed chromosomes [5,6]. This led to a model for DM segregation involving 'hitch-hiking' of DMs on chromosomes [6]. The distinctive clustering behavior of DMs and their close association with normal chromosomes (Figures 7,8) provide one mechanism by which DMs are transmitted to daughter cells even though they lack functional centromeres. Clustered DMs appear to transmit to daughter cells in a highly stochastic manner, however. As COLO320DM cells grow faster when their levels of c-*myc* expression are elevated [4], daughter cells containing more DMs should be selected, and over time the population of cells will come to contain many DMs per cell. Therefore, although the number of DMs per cell is continuously changing by stochastic uneven distribution to daughter cells, DMs appear to be stably maintained when one considers the entire population. Sometimes, clustered DMs associate with both groups of the segregating daughter chromosomes, forming bridges across the midplane of anaphase cells (Figure 7b). It is tempting to speculate that such chromosomal bridges could increase chromosomal instability by inducing non-disjunction or, in some cases, by preventing chromosome segregation and increasing the probability of a chromosome arm being severed during cytokinesis. Were breakage to occur within an arm, genomic instability could be further increased by inheritance of the broken chromosome, which could initiate a bridge-breakage fusion cycle [32].

The mechanism of DM clustering during mitosis remains to be elucidated. One possibility is that DMs cluster due to their interaction with the spindle microtubules, as interaction of non-centromeric chromatin with the mitotic spindle is well documented [33]. Chromosome spreads of colcemid-treated cells reveal a scattered distribution of DMs (Figure 7c), suggesting that spindle microtubules may play an important role in DM clustering. Alternatively, DMs themselves may have cohesive properties and may 'stick' to each other. It has been reported that a hamster cell line containing large tandemly repeated amplicons including the dihydrofolate reductase gene also had anaphase-bridge formations due to delayed sister-chromatid disjunction [34]. Repeated arrays of amplicons in DMs may have configurations that favor DM clustering and delayed disjunction of sister minute chromosomes during anaphase. The interaction between DMs and normal chromosomes must be clarified as well. The H2B–GFP system described here should facilitate the additional analyses required to understand the precise mechanism of DM clustering and segregation.

Conclusions

We have established a novel system for labeling chromatin in living cells using a fusion protein of histone H2B and GFP. The H2B–GFP system allows chromosomes, including DMs, to be imaged at a high resolution without perturbing cell cycle control or intracellular structures. The application of this system has revealed the distinctive clustering behavior of DMs in living mitotic cells. We propose that DM clustering is an important factor leading to their asymmetric distribution to daughter cells.

Materials and methods

Construction of H2B–GFP expression vectors

A human *H2B* gene was obtained by PCR amplification of human placental genomic DNA using primers which introduce *Kpn*I and *Bam*HI sites at the ends of one of the reported H2B sequences (GenBank accession number X00088) [35]: primer 1, 5′-CGGGTACCGCCACCATGCCAGAGCCAGCGAAGTCTGCT-3′; primer 2, 5′-CGGGATCCTTAGCGCTGGTGTACTTGGTGAC-3′. Primer 1 introduced the Kozak consensus sequence in front of the initiation codon. PCR reaction parameters were as follows: 95°C for 10 min, 25 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, followed by 72°C for 5 min. One of the obtained clones, which was 98.4% identical to the H2B gene (X00088), was used to construct the H2B–GFP vector. The PCR product was digested with *Kpn*I and *Bam*HI and subcloned into the cloning site of pEGFPN1 vector (Clontech) [18]. The H2B–GFP chimeric gene was subcloned into a mammalian expression vector (J. Kolman, T.K., unpublished observations). H2B–GFP expression was driven by the EF-1α promoter, a strong promoter in mammalian cells [36]. The vector contained the blasticidin resistance gene [37] as a selection marker. A retroviral vector, pCLNC-H2BG, was constructed by cloning the H2B–GFP gene into the pCLNCX vector [38]. Cloning details are available upon request.

Cell lines and transfection

HeLa cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Exponentially growing cells (in 10 cm dishes) were transfected with 20 μg H2B–GFP expression vector using a calcium phosphate precipitation protocol [39]. Transfected cells were replated 48 h after transfection and 5 μg ml^{-1} blasticidin-S (Calbiochem) was added 72 h after transfection. Five days later, the medium was changed to 2 μg ml^{-1} blasticidin-S. After 15 days of drug selection, surviving

colonies were checked under fluorescence microscopy and GFP-positive colonies were isolated. Several clones were selected and expanded into cell lines for further analyses. Production of VSV-G pseudotyped retrovirus [23] was performed by co-transfection of pCLNC-H2BG and pMD.G (the plasmid encoding the envelope protein VSV-G) into 293 gp/bsr cells as described [40]. COLO320DM cells [24] were grown in RPMI1640 medium supplemented with 10% FCS. Exponentially growing cells (in 10 cm dishes) were incubated with the concentrated viral supernatants in the presence of 8 μg ml^{-1} Polybrene (Aldrich). After overnight incubation, medium was changed and the infected cells were expanded for further analyses without drug selection.

Mononucleosome preparation

Mononucleosomes were purified according to the protocol (kindly provided by T. Ito and J. Kadonaga) [19] with several modifications. HeLa cells and stable cells expressing H2B–GFP (3×10^7) were trypsinized, harvested and washed once with 1× RSB buffer (10 mM Tris pH 7.6, 15 mM NaCl, 1.5 mM $MgCl_2$). After centrifugation, the cell pellet was resuspended in 1× RSB buffer with 1% Triton-X 100, homogenized by five strokes with a loose-fitting pestle to release nuclei. Nuclei were collected by centrifugation and washed twice with 1 ml buffer A (15 mM Tris pH 7.5, 15 mM NaCl, 60 mM KCl, 0.34 M sucrose, 0.5 mM spermidine, 0.15 mM spermine, 0.25 mM PMSF and 0.1% β-mercaptoethanol). Nuclei were finally resuspended in 1.5 ml buffer A and 15 μl 0.1 M $CaCl_2$ was added.

For making nucleosomal ladders, 0.5 ml suspended nuclei were digested by adding 1 μl micrococcal nuclease (Sigma, 200 units ml^{-1}) at 37°C. Aliquots (60 μl) were taken at each time point (1, 5, 10, 15, 30, 60 min) and 1.5 μl of 0.5 M EDTA was added to stop the reaction. To each tube, 18 μl H_2O, 12 μl 10% SDS and 24 μl 5 M NaCl were added. The mixtures were extracted with phenol–chloroform and 5 μl supernatant was analyzed by 1.5% agarose gel electrophoresis.

Limit digests for making mononucleosomes were performed by adding 10 μl micrococcal nuclease (200 units ml^{-1}) to 1.0 ml suspended nuclei. After 2 h digestion at 37°C, 20 μl 0.5 M EDTA was added to stop the reaction. The digest was centrifuged at 10,000 rpm for 10 min and the supernatant was collected. The pellet (chromatin fraction) was resuspended in 450 μl 10 mM EDTA, 50 μl 5 M NaCl was added to solubilize it, and the mixture was centrifuged at 14,000 rpm for 5 min to remove debris. The chromatin fraction was further fractionated on a 5–30% sucrose gradient for 18 h at 26,000 rpm in a Beckman SW41 rotor. After centrifugation, 1 ml fractions were collected and small aliquots (50 μl) of each sample were taken for DNA analyses. The remainder of each sample (950 μl) was precipitated with 280 μl 100% TCA with deoxycholic acid and left on ice for 10 min. The samples were then centrifuged at 3000 rpm for 5 min and each pellet was washed with acetone followed by a 70% ethanol wash. The pellet was air dried and resuspended in 20 μl of 1× SDS sample buffer, and aliquots were analyzed by gel electrophoresis through SDS–15% polyacrylamide gels followed by Coomassie staining. Western blotting was performed using anti-human-H2B antibody (1:1000, Chemicon) as a primary antibody. Signals were detected by enhanced luminol reagents (NEN Life Science Products) according to the manufacturer's instructions.

FACS analyses

HeLa cells and HeLa cells expressing H2B–GFP were harvested by trypsinization, fixed in 70% ethanol for 3 h at 4°C. Cells were stained with PI (20 μg ml^{-1}) containing RNase (200 ng ml^{-1}). Fluorescence was measured using a FACScan (Becton Dickinson). The red (PI) and green (GFP) emissions from each cell were separated and measured using standard optics. Color compensation was done to eliminate the artifact due to the overlap of PI and GFP emission. Cell debris and fixation artifacts were gated out. Data analysis was done using Cell Quest software (Becton Dickinson), and G1, S, and G2/M fractions were quantified using Multicycle software (Phoenix flow systems).

Fluorescence microscopy

For chromosome spreads, HeLa cells expressing H2B–GFP were treated with colcemid (100 ng ml^{-1}) for 1 h, trypsinized, harvested, and resuspended in hypotonic buffer (10 mM Tris pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$; 1.5×10^6 cells ml^{-1}) for 10 min. Swollen cells (50 μl) were attached to poly-L-lysine coated slide glasses by cytospin (90 sec), fixed in 3.7% formaldehyde for 5 min, 0.1% NP40 in phosphate-buffered saline for 10 min and counterstained with DAPI (1 μg ml^{-1}). Images were collected with a Nikon fluorescence microscope equipped with either DAPI or fluoroscein isothiocyanate (FITC) filter sets.

Immunofluorescence with a human anti-centromere antiserum was performed as described previously [41]. Microscopy was performed on a BioRad 1024 confocal microscope built on a Zeiss Axiovert 100 using a 63× 1.4 NA Zeiss Plan Achromat objective lens.

FISH was performed as previously described using a biotinylated c-*myc* cosmid probe and FITC–avidin [25]. For *in situ* fixation, asynchronously growing COLO320DM cells were directly fixed on chamber slides (Lab-Tek) without colcemid treatment as described [31].

To visualize H2B–GFP in living cells, cells were grown on 25 mm coverslips and mounted with prewarmed culture medium in a Dvorak–Stotler chamber (Lucas-Highland Company). Images were collected on the BioRad 1024 confocal microscope described above using either the 63× lens or a 40× 1.3 NA Neofluar objective using a laser power of 0.3–1% for GFP fluorescence. Transmitted light images were collected with DIC optics. Fluorescence images were overlaid onto DIC images using Adobe Photoshop. Z-series images were collected and stereoscopic images were made using the BioRad software supplied with the microscope.

For time-lapse microscopy, cells were cultured in 35 mm glass-bottom culture dishes (Mat Tek Corporation). The culture medium was supplemented with 20 mM HEPES pH 7.3 and mineral oil was overlaid to cover the surface of the medium. The dishes were mounted on a Nikon inverted fluorescence microscope (Diaphot 300) equipped with a video camera (CCD72; MTI). The temperature of the medium was kept constant at 37°C using a heated stage. A 100× 1.3 NA oil-immersion lens was used for observation. Images were acquired using IP Lab spectrum software (Signal Analytics Corporation).

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Selective capture of acentric fragments by micronuclei provides a rapid method for purifying extrachromosomally amplified DNA

Noriaki Shimizu[1,2], Teru Kanda[1], & Geoffrey M. Wahl[1]

The amplification and overexpression of a number of oncogenes is strongly associated with the progression of a variety of different cancers. We now present a strategy to purify amplified DNA on double minute chromosomes (DMs) to enable analysis of their prevalence and contribution to tumourigenesis. Using cell lines derived from four different tumour types, we have developed a general and rapid method to purify micronuclei that are known to entrap extrachromosomal elements. The isolated DNA is highly enriched in DM sequences and can be used to prepare probes to localize the progenitor single copy chromosomal regions. The capture of DMs by micronuclei appears to be dependent on their lack of a centromere rather than their small size.

Loss of genetic stability during cancer progression is a recurrent theme in oncogenesis. More than 50% of primary biopsy specimens and cell lines contain chromosomal aberrations that probably arose from defective recognition or repair of local DNA lesions, or from aberrant control of cell cycle transitions. As one example, cell cycle control mechanisms prevent gene amplification from occurring at measurable frequencies in normal cells — in contrast, amplification occurs in a substantial fraction of cancer *in vivo*[1–5].

Gene amplification can often, but not always, be detected cytogenetically. It is manifested microscopically as paired extrachromosomal acentric chromatin bodies called double minute chromosomes (DMs)[3,6], dicentric chromosomes[7–9], or variously sized chromosomal expansions exhibiting abnormal banding patterns such as homogeneously staining regions (HSRs)[6,10,11]. It is reasonable to assume that overexpressed amplified sequences contribute a selective or survival advantage since oncogene amplification correlates with a poor prognosis for patients with ovarian cancer (*HER-2 / neu*), breast cancer (c-*myc*, *HER-2 / neu*), neuroblastoma (N-*myc*) or small cell lung carcinoma (c-*myc*)[12]. In addition, elimination of extrachromosomally amplified drug resistance genes and oncogenes from rodent and human tumour cell lines restored drug sensitivity and decreased tumourigenicity, respectively[13–17]. If DMs in tumours encode genes that are rate limiting for cell growth *in vivo*, as they appear to be *in vitro*[13–15,18], then their elimination would provide a chemotherapeutic strategy targeted at a specific molecular defect uniquely found in cancer cells[19,20]. This is an attractive possibility as DMs occur frequently in clinically important neoplasms including those of the breast[21], lung[22], ovary[23], and colon[24].

Only a fraction of DMs or HSRs have been shown to contain known oncogenes. This is due to both the difficulty in obtaining adequate cytogenetic preparations and a lack of appropriate probes. The need for obtaining additional probes is demonstrated by recent comparative genomic hybridization (CGH)[4,5,25] and microdissection[26,27] analyses showing that a substantial fraction of breast cancers contain amplified sequences not detected by available molecular clones. While CGH and microdissection are yielding important insights into gene amplification and identifying new candidate oncogene loci, there is a growing need for a rapid, readily available strategy for isolating extrachromosomally amplified sequence.

We reported previously that DMs appear to be selectively entrapped in micronuclei during treatment with low concentrations of hydroxyurea (HU)[13]. This observation led us to develop the rapid and general method for micronucleus purification described herein. We show that purified micronuclei contain DNA highly enriched in DM sequences. This allows preparation of probes for fluorescence *in situ* hybridization (FISH) to identify the normal chromosomal regions from which the DMs were generated. Furthermore, we show that selective inclusion of DMs in micronuclei is not related to their small size. Rather chromosomes are excluded from micronuclei because of the presence of a centromere or some other feature contributing to normal chromosome function.

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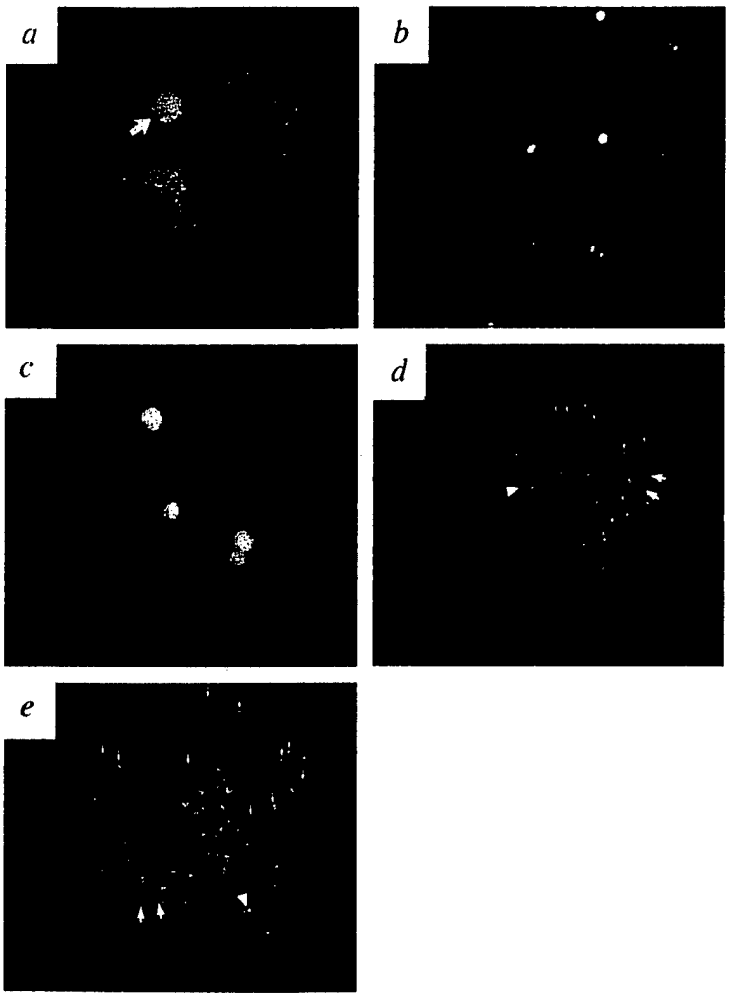


Fig. 1 Analysis of micronuclei and micronuclei-DM probes from COLO 320DM cells. *a*, Metaphase spread from COLO 320DM cells treated with 100 μM HU for 3 days hybridized to biotinylated c-*myc* cosmid probe and detected by FITC-avidin. The signal was amplified by a second layer of biotinylated anti-avidin and FITC-avidin. Micronucleus stained by the c-*myc* probe is shown by the arrow. *b*, *c*, Purified micronuclei from COLO 320DM cells were fixed and analysed by FISH as in (*a*). (Magnification of *b* was 400 ×, while all other photographs in this paper are 1,000 ×.) *d*, DNA in the purified micronuclei was uniformly amplified, FITC-labeled and hybridized to metaphases from COLO 320DM cells. Some DMs stained by the probe are indicated by small arrows. The three chromosomal regions stained by this probe are indicated by large arrows and an arrow head. *e*, For comparison to (*d*), a COLO 320 DM metaphase hybridized with c-*myc* cosmid probe. The signal was amplified as in (*a*). The same three chromosomal regions as in (*d*) are indicated by arrows and arrow head.

Purification of hydroxyurea-induced micronuclei

HU inhibits ribonucleotide reductase, thereby halting cell cycle progression in S phase when used at high concentration. Lower concentrations permit DNA replication but induce micronuclei[13]. HU effectively induces COLO 320DM cells to produce micronuclei that entrap the extrachromosomally amplified DNA at high efficiency (Fig. 1*a*). Therefore, we used COLO 320DM cells[28,29] to develop a DM purification strategy based on micronucleation.

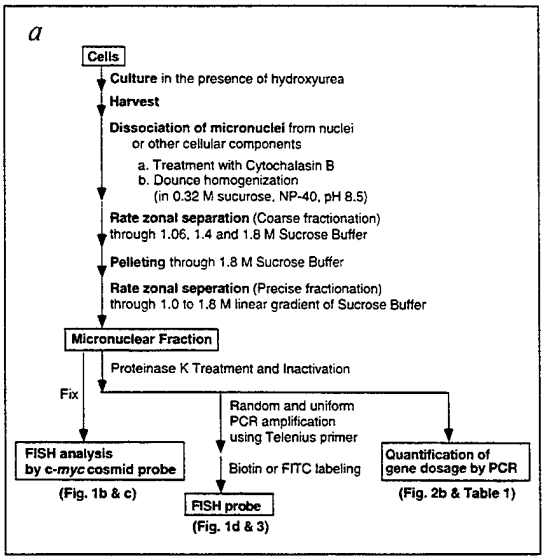
The micronucleus purification method is described in Fig. 2*a*. It reproducibly gives micronuclei in a higher yield and of greater purity than we could achieve using previously described methods designed to isolate microcells for use in monochromosome transfer experiments[30]. We first determined the HU concentration producing maximal yield of micronuclei with minimal induction of apoptosis. This involved 3 day treatment of COLO 320DM cells with 100 μM HU. We then attempted to dissociate micronuclei from nuclei using citric acid treatment followed by separation using flow cytometry as described previously[31]. However, this protocol caused severe aggregation of micronuclei during subsequent purification steps. Efficient dissociation of micronuclei from nuclei was accomplished by treating the cells with cytochalasin B to destroy actin filaments before and during homogenization, followed by increasing the pH of the lysis buffer to pH 8.5 to destroy intermediate filaments[32]. We purified micronuclei in the homogenate through the following 3 steps; 1) coarse separation by velocity sedimentation to remove most nuclei, 2) centrifugation through a 1.8 M sucrose layer to remove cytoplasmic components, and 3) fractionation by velocity sedimentation to completely remove nuclei. A high concentration of sucrose was included in each buffer to aid in the separation of micronuclei and to prevent their aggregation. Furthermore, we avoided wash steps to minimize loss of micronuclei.

The data in Fig. 1 and below show that the DNA in the final micronucleus preparation was highly enriched for DM sequences. We did not detect a single intact nucleus among thousands of micronuclei. More than 90% of the 4,6-diamino-2-phenylindole (DAPI)-positive particles were micronuclei based on their size and shape. Some debris was observed that was devoid of DNA as it failed to stain with DAPI. FISH using a c-*myc* cosmid probe showed that more than 80% of the RNase-treated, propidium iodide (PI)-positive structures exhibited intense hybridization (Fig. 1*b* and *c*), indicating the successful purification of micronuclei having DMs and/or submicroscopic DM precursors (episomes[33]).

Estimating micronucleated DM preparation purity

The purity of the COLO 320DM micronuclei preparation was measured using competitive PCR[34,35]. The amount of DM sequences (c-*myc*) relative to a single-copy chromosomal control sequence (β-*globin*) was estimated using an internal control for each. A fixed amount of test DNA (derived from genomic DNA or micronuclei) was amplified along with serially diluted internal standard DNA in the same tube using a single primer pair for each. We then determined the dilution of standard DNA yielding equal amounts of product from test DNA. DNA standards were prepared using human c-*myc* or β-*globin* primers to amplify a fragment of the desired size from a non-homologous DNA source (see Methods). This generated standard DNA and test DNA products differing slightly in size that could be amplified using a single pair of primers.

Our data show that DNA from human WS1 diploid cells competed almost equally with the c-*myc* or β-*globin* standard DNAs (Fig. 2*b*), indicating that these two genes exist in equal copy numbers in the WS1 genome. On the other hand, DNA from COLO 320DM cells competed with the c-*myc* standard about 60-fold more efficiently than the β-*globin* standard DNA with its cognate test DNA. This result implies that c-*myc* is amplified approximately 60-fold relative to β-*globin*, which compares favorably to the reported value for c-*myc* amplification in this cell line determined by quantitative Southern blotting (30- to 60-fold)[14,28]. Purified



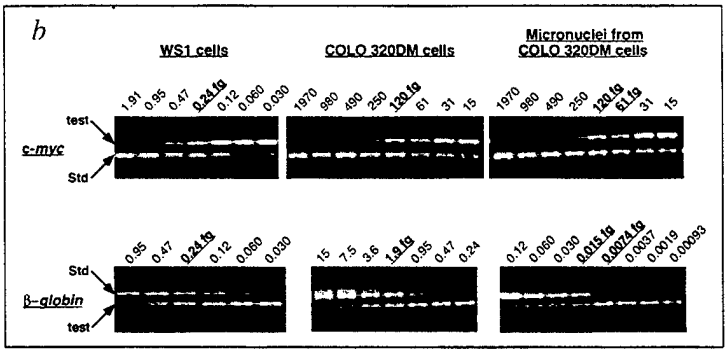


Fig. 2 Purification of micronuclei from COLO 320DM cells. *a*, Outline of the micronucleus purification procedure. *b*, Purity of DM sequences in the purified micronuclei. The amount of c-*myc* and β-*globin* sequences in total genomic DNA from WS1 or COLO 320DM, or from purified COLO 320DM micronuclei was quantified by competitive PCR. A constant amount of test DNA was amplified using c-*myc* or β-*globin* primer pairs in the presence of serially diluted standard DNAs. PCR products were fractionated by agarose gel electrophoresis and stained with ethidium bromide. The amount of standard DNA added to each reaction is noted above each lane. The numbers are bolded and underlined when the test and standard DNA had roughly equivalent band intensities.

micronuclear DNA from COLO 320DM cells was then subjected to competitive PCR amplification using either c-*myc* or β-*globin* primers. The DNA from micronuclei competed with the c-*myc* standard DNA about 8,000-fold more efficiently than with the β-*globin* standard DNA. This corresponds to a 128-fold enrichment of c-*myc* sequences.

Generality of procedure for enrichment of DM DNA

We examined the generality of the procedure by applying it to three additional tumour cell lines containing 4 to 16-fold amplification of c-*myc* genes. The average number of DMs per metaphase in all cell lines tested thus far ranged from a high of 44 ± 28 in COLO 320DM to a low of 3.3 ± 2.4 in the glioblastoma cell line D566 (ref. 36) (Table 1). This range encompasses that reported in many biopsy samples of human tumours[3]. Furthermore, the DMs in the medulloblastoma D425 (ref. 37) and in D566 were extremely small, and could barely be visualized using fluorescence microscopy of RNase treated and PI stained preparations. We treated these cell lines with 100 μM HU for 3 days and purified micronuclei as described for COLO 320DM cells. DNA from each cell line was analysed by the competitive PCR method described above to assess the fold enrichment achieved by the micronucleation procedure. Table 1 shows that the micronucleus purification protocol produced c-*myc* enrichments ranging from 32 to 128-fold. It is important to emphasize that the micronucleation protocol employed was that derived for COLO 320DM, and was not optimized individually for each cell line. These data indicate that the protocol described in Fig. 2*a* can be applied to a broad diversity of cell lines derived from tumour types including promyelocytic leukaemia, medulloblastoma, glioblastoma and neuroendocrine.

Use of purified micronuclear DNA for FISH

An important goal of DM purification is the isolation of DNA of sufficient quality and quantity to prepare FISH probes to enable identification of the chromosomal location(s) that generated the DMs. To investigate whether the micronuclear DNA could be used for FISH, it was uniformly amplified by degenerate oligonucleotide-primed-PCR (DOP-PCR) to produce probes[38]. The specificity of the probe generated from COLO 320DM micronuclei was analysed in two ways. It was first hybridized *in situ* to metaphase spreads of COLO 320DM cells. Fig. 1*d* shows significant hybridization to DMs, to chromosomal sites near the centromeric region of a medium sized chromosome, and to opposite arms of a single metacentric chromosome suggestive of a large chromosomal inversion. These three regions were identical to those stained by the c-*myc* cosmid DNA probe (Fig. 1*e*). Other chromosomal regions were essentially devoid of signal even if the signal was amplified using avidin-biotin sandwiches (data not shown). The probe generated from purified micronuclei DNA also brightly stained micronuclei formed in interphase COLO 320DM cells (data not shown).

We used the PCR amplified probes generated from micronuclei derived from COLO 320DM and the other cell lines listed in Table 1 to localize the corresponding sequences in metaphase chromosomes isolated from normal human peripheral blood lymphocytes (Fig. 3*a-d*). The probe from each cell line hybridized solely to the terminus of the long arm of a medium size sub-

Table 1 Enrichment of DM-sequence in purified micronuclei

Cell line	Tumour type	No. of DMs per metaphase[a]	Copy No. of c-*myc* relative to β-*globin*[b]		Fold enrichment
			Whole cells	Purified micronuclei	
COLO 320DM	Colon Carcinoma (Neuroendocrine)	43.6 ± 27.6	64	8,192	128
HL-60	Promyelocytic Leukaemia	4.9 ± 7.3	16	1,024	64
D425	Medulloblastoma	14.9 ± 11.4	8	256	32
D566	Glioblastoma	3.3 ± 2.4	4	256	64

[a]The number of DMs in RNase treated and PI stained metaphase was counted and expressed as mean ± standard deviation
[b]Copy numbers were determined in the same way as Fig. 2*b*

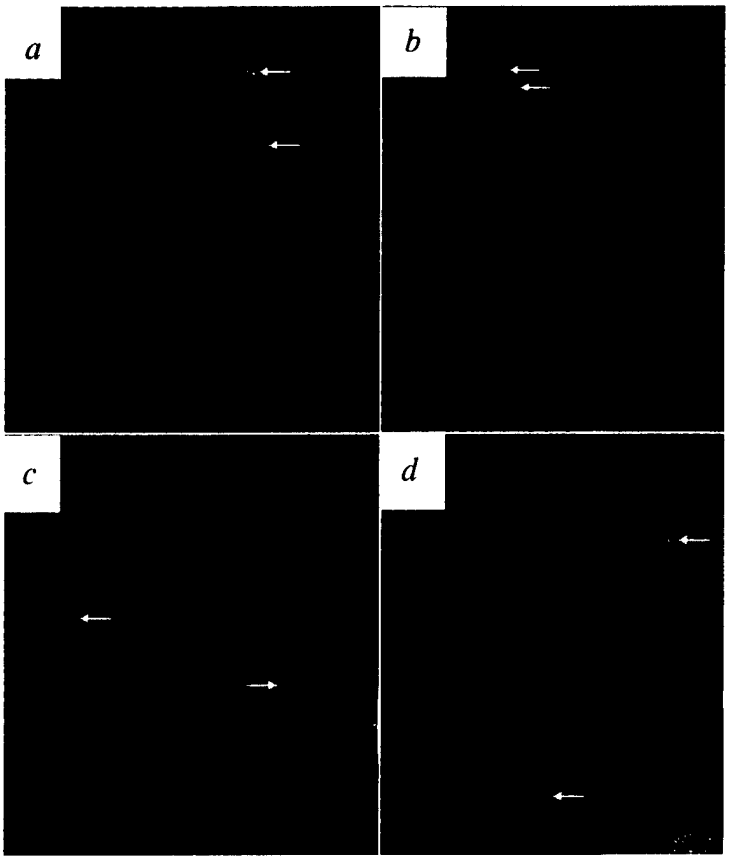


Fig. 3 Metaphases from normal human peripheral blood lymphocytes hybridized to the micronuclei probe from 4 different human tumour cell lines. Biotinylated probes were made from purified micronuclei of *a*, COLO 320DM cells; *b*, HL-60 cells; *c*, D425 cells; and *d*, D566 cells, and were hybridized to metaphases from normal human peripheral lymphocytes. The signal was amplified as in Fig. 1*a*. All probes reproducibly detected one locus (arrows) consistent with the known location of c-*myc* on 8q24.

metacentric chromosome. This position is consistent with the known location of c-*myc* (8q24)[39,40]. Thus, the enrichment obtained from each cell line was sufficient to produce a highly specific FISH probe capable of detecting the single copy locus from which the DMs were generated. The data further reveal that the DMs in each cell line contain sequences derived from only a single chromosomal location.

Centromeric excluded minichromosomes

The data presented above and elsewhere[13,16] show that DMs are preferentially captured by micronuclei. This raises the question of the mechanism underlying their apparently selective inclusion. We have begun to explore this issue by determining whether a minichromosome that is the approximate size of a typical DM is incorporated efficiently into micronuclei. We employed the CHO hybrid XEW8.2.3 (refs 41, 42) which contains a 1,000–2,000 kb minichromosome derived from the centromeric region of human chromosome 1. This minichromosome has a functional centromere as it exhibits high mitotic stability and is detected by a human centromere-specific probe (Fig. 4*a*).

XEW8.2.3 cells were treated with several doses of HU for 3 or 7 days to assess micronucleation frequency. Many micronuclei were induced by HU in a dose dependent manner (Fig. 4*b* and *c*). Hybridization of cell preparations showed that more than 95% of nuclei exhibited a single intense hybridization signal, attesting to the high efficiency of detection afforded by this probe (Fig. 4*b*). By contrast, few if any micronuclei contained the minichromosome of human centromere origin (Fig. 4*b* and *c*). These data indicate that size is not an important determinant of whether a genetic element can be incorporated into micronuclei. Rather, the results indicate that centromeric and/or other sequences involved in chromosome segregation or subnuclear localization may exclude small DNA fragments from micronuclei.

Discussion

Several useful methods have been developed to elucidate amplified sequences in tumour biopsy samples[25]. CGH can reveal the chromosomal locations from which amplified sequences in tumour cells arose[4,5], but it does not differentiate sequences amplified in DMs from those amplified within chromosomes. This is important for determining which tumours may benefit from therapeutic strategies that eliminate extrachromosomally amplified sequences. CGH may miss detecting amplified sequences in cases where amplicon size is small and copy number is low, and if the biopsy sample is contaminated with normal cells. Another method requires mechanical isolation of DMs from metaphase preparations of tumour tissue[26,27]. This method requires specialized equipment and technical expertise, as well as good quality metaphase spreads which are typically difficult to obtain from biopsy specimens. Microdissection is also restricted to microscopically visible DMs, and cannot be used to obtain submicroscopic episomes known to be present in many cell lines[33]. The isolation of only a few representative DMs among the many present in a tumour cell population may also produce an underestimate of the heterogeneity in the DM population. The method reported here avoids the problems inherent in other strategies by using micronuclei. This strategy bypasses the need for cytogenetics to obtain DNA highly enriched for DMs or episomes that can then be used as a FISH probe. As the procedure requires straightforward centrifugation protocols, it is accessible to most clinical and basic research laboratories. These potential advantages provide incentive to apply the method to tumour biopsy specimens, and the following discussion offers encouragement for its feasibility.

The application of the DM enrichment procedure to human tumours requires several conditions to be fulfilled. First, as biopsy specimens are often contaminated with normal cells, the presence of the latter must not adversely affect the DM enrichment achieved by the micronucleation procedure. We have found that mixing COLO 320DM cells with a 100-fold excess of normal cells still enables preparation of micronuclei that generate a probe that reacts intensely with the amplified sequences (data not shown). This is expected as normal cells typically produce micronuclei at very low efficiencies[43,44] which is not increased by HU treatment (N.S. *et al.*, manuscript in preparation). Second, it must be possible to induce diverse cell types to produce micronuclei with an acceptable frequency. Our data show that the micronucleation procedure

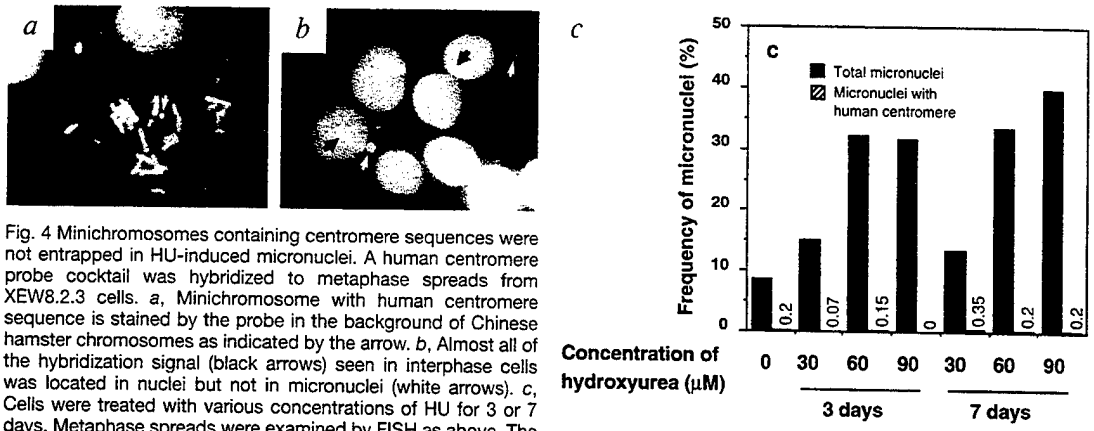


Fig. 4 Minichromosomes containing centromere sequences were not entrapped in HU-induced micronuclei. A human centromere probe cocktail was hybridized to metaphase spreads from XEW8.2.3 cells. *a*, Minichromosome with human centromere sequence is stained by the probe in the background of Chinese hamster chromosomes as indicated by the arrow. *b*, Almost all of the hybridization signal (black arrows) seen in interphase cells was located in nuclei but not in micronuclei (white arrows). *c*, Cells were treated with various concentrations of HU for 3 or 7 days. Metaphase spreads were examined by FISH as above. The numbers of total micronuclei (black bar) and the micronuclei stained by human centromere probe (hatched bar) were scored and expressed as frequency of micronuclei (%) relative to the number of interphase nuclei scored (more than 1000 for each point).

optimized for COLO 320DM cells can be applied to a leukaemia, medulloblastoma and a glioblastoma cell line. Third, sufficient enrichment must be achievable to allow preparation of a FISH probe from cell populations in which a minority of cells contain DMs, or where the amplification level is low. The enrichments we obtained using cell lines were sufficient to generate probes that hybridized intensely to the corresponding single copy c-*myc* locus.

Our observations concerning DM purification from cell lines representing four different tumour types suggest that this procedure might be extended to human tumour biopsy specimens containing cells with a low DM incidence and amplification level. All tumour cell lines we have analysed produce micronuclei even when they are not challenged with HU. Thus, it may be possible to obtain sufficient micronuclei directly from biopsy specimens to allow preparation of PCR-generated FISH probes. Alternatively, short term culture in the presence of HU might suffice to generate the required number of micronuclei.

The fold enrichment relative to a single copy chromosomal standard ranged from 32–128 fold. The difference in apparent enrichment may be due to the use of a single protocol for micronucleation which was not optimized for cell lines other than COLO 320DM. As the precise amount of DNA which is amplified in each cell line is not known, it is difficult to provide an accurate estimate of the absolute purity of each preparation.

The mechanisms accounting for the selective entrapment of DMs by micronuclei remain to be elucidated. Our data show that small size is not a determining factor. We consider two other alternatives to be most likely. First, the micronuclei induced by HU treatment resemble those induced by clastogenic agents in that they are highly enriched for acentric fragments[45–47]. It is possible that HU treatment generates DNA double-strand breaks at low efficiency by slowing replication fork progression, as reported for other drugs or genetic manipulation[48,49]. Micronuclei may be the result of reconstitution of the nuclear membrane around lagging chromosomal fragments at mitosis. A second model is that acentric fragments such as DMs reside in a different nuclear location than chromosomes. The presence of elements in ectopic nuclear environments may promote 'budding' of these sequences from interphase nuclei. Consistent with this, we have observed nuclei in which DM sequences appear to bud out of the nuclear membrane to form micronuclei (N.S. & G.M.W., unpublished observation). Our observation that small chromosomes are effectively excluded from HU-induced micronuclei could provide the basis for determining whether functional centromeres, telomeres, or both are required for exclusion from HU-induced micronuclei. This, in turn, could provide the basis for a facile assay for structure-function analyses of centromeres and telomeres.

Many of the genetic changes resident in each cancer cell genome reflect the various selections imposed on the cell during tumour progression. However, the contributions of specific alterations to tumour cell growth or survival *in vivo* remain to be elucidated, and it is uncertain how many changes detected in late stage tumours are of consequence. By contrast, the presence of acentric extrachromosomal molecules implies that one or more of the genes they encode confers a selective advantage or else they would be lost, as observed when drug selection is removed from cells containing DM-encoded drug-resistance genes[16]. The strategy described here can aid in both identifying genes encoded by DMs, and in revealing how known and previously unknown proto-oncogenes may contribute to particular stages of tumourigenesis.

Methods

Cell lines. Human COLO 320DM neuroendocrine tumour cell lines[28,29] were provided by D. D. Von Hoff (University of Texas, San Antonio, Texas) and grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). The locations of amplified c-*myc* genes to DMs was confirmed by FISH using c-*myc* cosmid DNA. Human HL–60 promyelocytic leukaemia cell line[50] was obtained from American Type Culture Collection (CCL240) and cultured in RPMI 1640 supplemented with 10% FCS. Human D425 medulloblastoma cell line[37] and human D566 glioblastoma cell line[36] were provided by S.H. Bigner (Duke University Medical Center), and maintained in improved MEM Zinc Option Medium (Gibco) supplemented with 10 mM HEPES buffer and 10% FCS. The XEW8.2.3 cell line[41,42] was isolated by I. Scheffler (University of California, San Diego, California) and maintained in DMEM supplemented with 10% FCS. Human WS1 diploid fibroblasts were obtained from American Type Culture Collection (CRL1502) and maintained in DMEM supplemented with 10% heat-inactivated FCS and 1× MEM nonessential amino acids. All cells were grown at 37 °C with 7% CO_2. HU (Sigma) was added to

the culture when passaged. Cells were grown in the presence of 100 μM HU for 3 days (unless otherwise noted).

Purification of micronuclei. Cells ($1 \times 10^8 \sim 1 \times 10^9$ cells) grown in the presence of 100 μM HU for 3days were harvested and washed twice with DMEM without serum. The cell pellet was resuspended in 20 ml of prewarmed (37 °C) DMEM containing cytochalasin B (10 μg/ml) and incubated for 30 min at 37 °C. After collection by centrifugation at 200 × g for 5 min, the cells were resuspended in 10 ml pre-chilled lysis buffer (10 mM Tris-HCl, 2 mM Mg-acetate, 3 mM $CaCl_2$, 0.32 M sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% (v / v) Nonidet P–40, 0.15 mM spermine, 0.75 mM spermidine and 10 μg/ml cytochalasin B, pH 8.5, 4 °C), and Dounce-homogenized (5 to 10 strokes using a loose-fitting pestle). The release of micronuclei from other cellular components was confirmed by mixing a small portion of the homogenate with an equal volume of phosphate buffered saline without divalent cations (PBS-) containing 2 μg/ml of DAPI and examining under a fluorescence microscope. The homogenate was mixed with an equal volume of 1.8 M sucrose buffer (10 mM Tris-HCl, 1.8 M sucrose, 5 mM Mg-acetate, 0.1 mM EDTA, 1 mM dithiothreitol, 0.3% BSA, 0.15 mM spermine, 0.75 mM spermidine, pH 8.0, 4 °C), and a 10 ml portion was layered on top of the layers of sucrose buffers (20 and 15 ml containing 1.8 M and 1.6 M of sucrose, respectively) in a 50 ml-tissue culture tube. This was centrifuged in a JS–5.2 swinging bucket rotor (Beckman) at 2,000 rpm (gmax = 944) for 20 min at 4 °C. After centrifugation, fractions were collected from the top of the tube and examined by DAPI staining as described above. The upper 3 ml of the gradient usually did not contain micronuclei and was discarded. Micronuclei were recovered from the next 12 ml. Although most nuclei were pelleted, a significant amount of nuclear contamination was seen in this fraction. The fraction was layered on a 5 ml cushion of 1.8 M Sucrose Buffer and centrifuged at 14,000 rpm (gmax = 34,700) for 90 min at 4 °C using the SW40 rotor (Beckman). The supernatant was removed completely, and the pellet containing micronuclei and contaminating nuclei was collected and resuspended in 0.2 ml of 0.8 M Sucrose Buffer. The suspension was then layered on top of an 11 ml linear sucrose gradient (1.0 to 1.8 M in Sucrose Buffer) made in a 15-ml tissue culture tube, and centrifuged at 1,500 rpm (gmax = 530) for 15 min at 4 °C using JS–5.2 rotor. After centrifugation, fractions (1 ml each collected from the top) were examined by DAPI staining. More than 90% of the DAPI-stained particles seen in fractions 1 to 4 were micronuclei of various sizes. Most of the nuclei pelleted under this condition, and absolutely no nuclear contamination was seen in the top four fractions. The micronuclei fractions were diluted 5-fold by adding PBS, then centrifuged at 2,000 rpm (gmax = 944) for 15 min at 4 °C using JS–5.2 rotor. The pellet was suspended in a small amount of buffer (~ 0.1 ml). A portion of the purified micronuclei was fixed in methanol / acetic acid (3 / 1), and examined by FISH using a c-*myc* cosmid probe. The remaining samples were treated with proteinase K (60 μg/ml) and 0.05% Triton X–100 for 60 min at 50 °C followed by inactivation of the enzyme at 94 °C for 12 min, and then used for gene quantification by PCR or the generation of FISH probes.

Gene quantification by PCR. We quantified the levels of c-*myc* gene amplification and DM enrichment relative to the single copy β-*globin* gene on chromosome 11 using competitive PCR[34,35]. The primers used for the c-*myc* gene were myc-C, 5'd(CTG GGA TCT TCT CAG CCT AT)–3' and myc-D, 5'd(ACT CCT CTC ACC ATG AAG GT)–3'. The sequences of primers used for β-*globin* are IVS-I, 5'd(GTA TCA TGC CTC TTT GCA CC)–3', and IVS-L, 5'd(AAG GGC CTA GCT TGG ACT CA)–3'. The primer set myc-C and myc-D amplifies 400 bps of the human c-*myc* gene intron 2, and the primer set IVS-I and IVS-L amplifies 214 bp of the human β-globin gene intron 2. Internal standards for each gene were generated from salmon DNA or *Saccharomyces pombe* DNA by PCR amplification using c-*myc* or β-*globin* primer pairs, respectively, by lowering the annealing temperature to 42 °C or 47 °C, respectively. The products were separated by agarose gel electrophoresis, and bands differing in size from the human DNA PCR product were excised. The excised bands were further purified by three successive rounds of PCR amplification at a higher annealing temperature (63 °C) and fractionation by agarose gel electrophoresis. The final PCR products, each of which gave a single band in agarose gel electrophoresis, were used as the internal standards in competitive PCR. Competitive PCR reactions contained equal amounts of test DNA and serial 2-fold dilutions of standard DNA. Each tube (10 μl) contained 1 × Taq buffer (Invitrogen; N for c-*myc*, J for β-globin), 0.2 mM of each dNTP, 20 ng of each primer, 0.2 ml test DNA, serially-diluted known amounts of standard DNA, and 0.4 U Taq DNA polymerase (Boehringer-Manheim). The tubes were heated to 95 °C for 3 min followed by 35 cycles at 94 °C for 1 min, 63 °C for 1 min, and 72 °C for 2 min. The products were then separated by agarose gel electrophoresis, stained with ethidium bromide and the intensities of the products from the test and standard DNA were compared.

FISH. DNA in the proteinase K-treated micronuclei preparation was uniformly amplified by randomly primed PCR[38]. Amplified products were labelled with biotin using the 'Bio-Prime DNA Labelling System' (BRL Life Technologies) as per the manufacturer's protocol. The experiment in Fig. 1*d* employed uniformly amplified products that were FITC-labelled by the 'Prime-It Fluor Fluorescence Labelling Kit' (Stratagene). c-*Myc* cosmid DNA was obtained from Y. Yin (Salk Institute)[13] and labelled with biotin–14-dCTP by random hexamer extension. Biotinylated human centromere probe cocktail was purchased from Oncor. Metaphase spreads were treated with RNase (100 μg/ml in 2× SSC, 37 °C, 60 min), and hybridized using standard conditions[51]. Briefly, for each hybridization, 50–100 ng of probe was used in 15 μl of hybridization mixture (containing 50% formamide, 10% dextran sulfate, 2× SSC, 6 μg salmon sperm DNA, 3 μg shared human placental DNA (only for the experiment of Fig. 3) and 3 μg human COT I DNA (BRL) (not included in the experiments of Fig. 4)) which was denatured at 75 °C for 5 min followed by 42 °C for 30 min. The slides with metaphase spreads were denatured in 70% formamide, 2× SSC at 72 °C for 2 min, dehydrated in ice cold 70%, 85% and 100% ethanol for 3 min each, and air dried. Hybridization was done at 37 °C in a moist chamber overnight. Slides were then washed three times in 50% formamide, 2× SSC at 45 °C for 3 min each, 3 × 3 min in 2× SSC at 45 °C, and one time in 0.1× SSC at 60 °C for 10 min. Slides were viewed at this point when FITC labelled probes were used. Alternatively, the hybridization signal of biotin labelled probe was detected with one layer of FITC-conjugated avidin (Vector), amplified with biotinylated anti-avidin (Vector) and a second layer of FITC-conjugated avidin. Slides were counterstained with 0.5 μg/ml of PI in Vectashield (Vector) and examined with a Zeiss fluorescence microscope equipped with the appropriate epifluorescence filters. All photographs were taken at a magnification of 1,000×, except Fig. 1*b* which was 400× as indicated in the legend.

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PII Redacted

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METHOD FOR ISOLATION OF EXTRACHROMOSOMAL AMPLIFIED GENES

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BACKGROUND OF THE INVENTION

1. *Field of the Invention*

This invention relates generally to gene amplification and specifically to a method for isolation of extrachromosomal amplified nucleic acid sequences.

2. *Description of Related Art*

Gene amplification in tumor cells results in the production of multiple copies of a genomic region. Amplification of oncogenes leads to the over expression of proteins participating in the transduction of growth-related signals and confers a growth advantage to tumor cells. Clinically, oncogene amplification is extremely common in human tumors and correlates with a poor prognosis for patients with ovarian cancer (HER-2/neu), breast cancer (c-myc, HER-2/neu), neuroblastoma (N-myc), or small cell lung cancer (c-myc) (Slamon, *et al., Science* 235:177, 1987; Slamon, *et al., Science*, 244:707, 1989; Seeger, *et al., N. Engl. J. Med.*, 313:1111, 1985; Johnson, *et al., J. Clin. Invest.*, 78:525, 1986). There is also evidence that amplification of drug resistant genes is associated with both *in vitro* and *in vivo* resistance of a patient's tumor to an antineoplastic agent (Schimke, R., *Cancer Res.*, 44:1735, 1984; Stark, G., *Cancer Surv.* 5:1, 1986; Trent, *et al., J. Clin. Oncol.*, 2:8, 1984; Curt, *et al., N. Engl. J. Med.*, 308:199, 1983).

Amplified genes have been localized to two types of cytogenetically distinguishable structures. These structures can be located on the chromosome, within homogeneously staining regions (HSRs), or they can be reside extrachromosomal either as submicroscopic elements called episomes or as larger structures called double minute chromosomes (Carroll, *et al., Mol. Cell. Biol.*, 8:1525, 1988; Von Hoff, *et al., J. Clin. Invest.*, 85:1887, 1990; Von Hoff, *et al., Proc. Natl. Acad. Sci. USA*, 85:4804, 1988). The occurrence of DMs in a malignant cell line was first described by Spriggs, *et al.* (*Br Med J*, 2:1431, 1962). DMs are paired, acentric fragments that segregate randomly at cell division and can be detected in the majority of primary tumors at biopsy (Benner, *et al., Anticancer Drugs*, 2:11, 1991; Gebhart, *et al., Int. J. Cancer*, 34:369, 1984). DMs tend to vary in size and also in number of DMs per cell.

Genes amplified on DMs can be lost spontaneously at each cell division or can be eliminated by treatment with hydroxyurea (HU) at concentrations that do not inhibit DNA synthesis or ribonucleotide reductase (Von Hoff, *et al., Proc. Natl Acad Sci, USA*, 89:8165, 1992; Von Hoff, *et al., Cancer Res.*, 51:6273, 1991). It appears that HU-treatment of cells containing DMs results in an increase in micronuclei formation, and the capture of the DMs within the micronuclei (Von Hoff, *et al., Proc. Natl Acad Sci, USA*, 89:8165, 1992; Von Hoff, *et al., Proc. Am Assoc. Cancer Res.*, 33:359, 1992). By contrast, HSRs are not lost during cell division or by treatment with HU and represent a stable form of gene amplification. Elimination *c-myc* genes contained in DMs from a colon cancer cell line of neuroendocrine origin (COLO 320 DM) reduced its tumorigenicity in nude mice (Von Hoff, *et al., Proc. Natl. Acad. Sci., USA*, 89:8165, 1992). Studies have shown that treatment of HL60 cells with low concentrations of HU reduced the number of *c-myc*-containing DMs, which led to decreased *c-myc* expression and induction of differentiation (Eckhardt, *et al., Proc. Natl. Acad. Sci., USA*, 91:6674, 1994; Shimizu, *et al., Cancer Res.*, 54:3561, 1994). The studies also showed that agents which eliminate extrachromosomal DNA also alter tumor phenotype.

Similarly, previous studies have shown that when the selective pressure of a drug is removed from mammalian cells that carry unstably amplified genes on extrachromosomal particles, the cells gradually lose those amplified genes (lose their DMS or episomes). Snapka and Varshavsky previously showed that HU could increase the rate of loss of unstably amplified dihydrofolate reductase (DHFR) genes from mouse cells (*Proc. Natl. Acad. Sci., USA*, 80:7533, 1983). Von Hoff, *et al.*, showed a similar elimination of the multidrug resistance gene 1 (MDR1) gene in vinblastine resistant human squamous tumor cells, as well as carbamylphosphate synthetase, aspartate transcarbamylase, dihydro-orotase (CAD) genes from N-(phosphonacetyl)-L-aspartic acid (PALA) resistant Chinese hamster ovary cells, and DHFR genes from methotrexate resistant human squamous tumor cells (*Cancer Research*, 51:6273, 1991).

The persistence of DMs implies that these acentric elements express one or more genes that impart a growth or survival advantage to the cell. Identification of the expressed nucleic acid sequences contained in such DMs would provide a means for developing appropriate diagnostic, prognostic and therapeutic strategies.

SUMMARY OF THE INVENTION

The knowledge that gene amplification occurs in cancer cells provides an unparalleled opportunity for developing therapeutic approaches that are highly specific for tumor cells. The ability to eliminate amplified genes by removal or selective interference with their expression is enhanced by the determination of the identity of the amplified gene. The present invention provides a method for isolating and for identifying amplified genes which exist extrachromosomally in DMs within a cell.

The method of the invention allows isolation and molecular cloning of target nucleic acid sequences contained in extrachromosomal amplified loci. The identity of such nucleic acids or genes, whether previously known or unknown, provides a means for more accurate diagnosis and prognosis for a subject having a disorder such as a cancer. The identity of the genes also provides a method for monitoring the course of therapy for such a subject.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows a flow chart for isolation of micronuclei and amplified nucleic acid sequences contained within the micronuclei.

FIGURE 2a shows the frequency of micronuclei having c-myc versus the total number of interphase nuclei in COLO 320DM cells.

FIGURE 2b shows the frequency of micronuclei in interphase nuclei in B-1/50 cells (mouse cells having amplification of adenosine deaminase gene).

FIGURE 2c shows the frequency of micronuclei in interphase nuclei in XEW 8.2.3 cells (CHO cells having human centromere sequences and minichromosome).

FIGURE 3 shows DNA in purified micronuclei from COLO 320DM cells (FITC labeled) and FISH hybridized to COLO 320DM metaphase spreads (x 1000 magnification).

FIGURE 4 shows micronuclei formed in COLO 320DM cells and FISH hybridized to c-myc cosmid DNA and detected by FITC (x1000 magnification).

FIGURE 5 shows purified micronuclei from COLO 320DM cells treated with HU (100 uM) for 3 days and FISH hybridized to c-myc cosmid DNA (x400 magnification).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for isolating extrachromosomal amplified nucleic acid sequences from a cell having or suspected of having a cell proliferative disorder. Such disorders are associated with the amplification of cell growth control genes, oncogenes, multidrug resistance genes, and growth factor receptors, for example. The method of the invention provides a means for the identification of both previously known and of unknown expressed amplified nucleic acid sequences. Once the amplified nucleic acid is isolated and identified, probes can be developed for use in diagnosis, prognosis, and for monitoring a particular therapeutic regime.

In a preferred embodiment, the invention provides a method for isolating an amplified target nucleic acid associated with a cell proliferative disorder comprising subjecting a cell suspected of having a cell proliferative disorder to conditions sufficient to produce micronuclei, isolating the micronuclei from the cell, amplifying target nucleic acid in the micronuclei, wherein the target nucleic acid is associated with a cell proliferative disorder, and isolating the amplified target nucleic acid.

The term "isolated" as used herein refers to polynucleotides substantially free of other nucleic acids, proteins, lipids, carbohydrates or other materials with which they are naturally associated. Polynucleotide sequences of the invention include DNA, cDNA and RNA sequences which encode amplified extrachromosomal target gene or loci.

The method of the invention includes subjecting a cell suspected of having a cell proliferative disorder to conditions sufficient to produce micronuclei. Micronuclei refer to structures which preferably entrap extrachromosomal nucleic acid molecules and only infrequently contain chromosomes. In the method of the invention, a cell is subjected to an agent which induces micronuclei formation. Such agents include, but are not limited to inhibitors of DNA replication, DNA damaging agents, inhibitors of topoisomerase II, and membrane disrupting agents. Examples of such agents which induce micronuclei

formation include hydroxyurea, retinoic acid, dimethyl sulfoxide, guanazole, etoposide, proflavine, and difluoromethylornithine. Other agents having the function of those described herein will be known to those of skill in the art.

Hydroxyurea is utilized in the method of the invention at a concentration of about 1μM to 200μM, preferably from about 50μM to 150μM and most preferably from about 75μM to 100μM. Methods for evaluating the effectiveness of agents for inducing micronuclei include preparation of metaphase chromosomes and interphase nuclei and fluorescent *in situ* hybridization (FISH), as described in the illustrative EXAMPLES herein.

Isolation of the micronuclei from the cell is accomplished by physical separation, density gradient separation and/or immunoseparation. Such methods will be known to those of skill in the art. Preferably, the physical separation is differential centrifugation. Density gradient separation may be utilizing a medium such as Ficoll® or Percoll® (registered trademarks of Pharmacia), sucrose, or bovine serum albumin. Other comparable density gradient separation medium will be known to those of skill in the art or will be readily ascertainable. (See for example, *Current Protocols in Molecular Biology*, Ausubel, ed., Wiley & Sons, 1994; Coligan, *et al.*, *Current Protocols in Immunology*, Wiley Interscience, 1994).

Immunoseparation is optional and is performed by means of an antibody having the specificity of an anti-nuclear pore antibody or an anti-lamin antibody. Other antibodies having the specificity of an anti-nuclear pore antibody or an anti-lamin antibody and which bind to micronuclei are also useful in the immunoseparation process.

Following isolation of micronuclei from a cell, amplification is accomplished by polymerase chain reaction or other comparable means of amplification typically utilized by those of skill in the art. Oligonucleotide primers are used according to the invention and are employed in any amplification process that produces increased quantities of target nucleic acid or target nucleic acid sequence. Typically, one primer is complementary to

the negative (-) strand of the nucleotide sequence and the other is complementary to the positive (+) strand. Annealing the primers to denatured nucleic acid followed by extension with an enzyme, such as the large fragment of DNA Polymerase I (Klenow) or Taq DNA polymerase and nucleotides or ligases, results in newly synthesized + and - strands containing the target nucleic acid. Because these newly synthesized nucleic acids are also templates, repeated cycles of denaturing, primer annealing, and extension results in exponential production of the region (*i.e.*, the target nucleotide sequence) defined by the primer which is a discrete nucleic acid duplex with termini corresponding to the ends of the specific primers employed. Those of skill in the art will know of other amplification methodologies which can also be utilized to increase the copy number of target nucleic acid.

Preferably, the method of amplifying is by PCR, as described herein and as is commonly used by those of ordinary skill in the art. Alternative methods of amplification have been described and can also be employed as long as the target nucleic acid amplified by PCR using suitable primers is similarly amplified by the alternative means. Such alternative amplification systems include self-sustained sequence replication, 3SR, which begins with a short sequence of RNA of interest and a T7 promoter. Reverse transcriptase copies the RNA into cDNA and degrades the RNA, followed by reverse transcriptase polymerizing a second strand of DNA. Another nucleic acid amplification technique is nucleic acid sequence-based amplification (NASBA) which uses reverse transcription and T7 RNA polymerase and incorporates two primers to target its cycling scheme. NASBA can begin and end with either DNA or RNA and finish with either, and amplifies up to 10^8 copies within 60 to 90 minutes. Alternatively, nucleic acid can be amplified by ligation activated transcription (LAT). LAT works from a single-stranded template with a single primer that is partially single-stranded and partially double-stranded. Amplification is initiated by ligating a cDNA to a promoter oligonucleotide and within a few hours, amplification is 10^8 to 10^9-fold. The Qβ replicase system can be utilized by attaching an RNA sequence called MDV-1 to RNA complementary to a DNA sequence of interest. Upon mixing with a sample, the hybrid RNA finds its complement among the specimen's

mRNAs and binds, activating the replicase to copy the tag-along sequence of interest. Another nucleic acid amplification technique, ligase chain reaction (LCR), works by using two differently labeled halves of a sequence of interest which are covalently bonded by ligase in the presence of the contiguous sequence in a sample, forming a new target. The repair chain reaction (RCR) nucleic acid amplification technique uses two complementary and target-specific oligonucleotides, thermostable polymerase and ligase, and DNA nucleotides to geometrically amplify targeted sequences. A 2-base gap separates the oligonucleotide probes, and the RCR fills and joins the gap, mimicking normal DNA repair. Nucleic acid amplification by strand displacement activation (SDA) utilizes a short primer containing a recognition site for *Hinc*II with a short overhang on the 5' end which binds to target DNA. A DNA polymerase fills in the part of the primer opposite the overhang with sulfur-containing adenine analogs. Following amplification, *Hinc*II is added to cut the unmodified DNA strand. A DNA polymerase that lacks 5' exonuclease activity enters at the site of the nick and begins to polymerize, displacing the initial primer strand downstream and building a new one which serves as more primer. SDA produces greater than 10^7-fold amplification in 2 hours at 37°C. Unlike PCR and LCR, SDA does not require instrumented temperature cycling. Although PCR is the preferred method of amplification of the invention, these other methods can also be used to amplify the Amplified target nucleic acid locus as described in the method of the invention.

Primers which can be used for amplification of the target DNA sequence in the method of the invention embrace oligonucleotides of sufficient length and appropriate sequence so as to provide specific initiation of polymerization of a significant number of nucleic acid molecules containing the target nucleic acid. In this manner, it is possible to selectively amplify the specific target nucleic acid sequence containing the nucleic acid of interest. Specifically, the term "primer" as used herein refers to a sequence comprising a suitable number of deoxyribonucleotides or ribonucleotides, preferably at least eight, which sequence is capable of initiating synthesis of a primer extension product, which is

substantially complementary to a target nucleic acid strand. The oligonucleotide primer typically contains 15-22 or more nucleotides, although it may contain fewer nucleotides.

Experimental conditions conducive to amplification include the presence of nucleoside triphosphates and an agent for polymerization, such as DNA polymerase, and a suitable temperature and pH. The primer is preferably single stranded for maximum efficiency in amplification, but may be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent for polymerization. The exact length of primer will depend on many factors, including temperature, buffer, and nucleotide composition.

Primers are designed to be "substantially" complementary to each strand of the nucleotide sequence to be amplified. Substantially complementary means that the primers must be sufficiently complementary to hybridize with their respective strands under conditions which allow the agent for polymerization to function. In other words, the primers should have sufficient complementarity with the flanking sequences to hybridize therewith and permit amplification of the nucleotide sequence. Preferably, the terminus of the primer that is extended has perfectly base paired complementarity with the complementary flanking strand.

Oligonucleotide primers for use in the invention may be prepared using any suitable method, such as conventional phosphotriester and phosphodiester methods or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage, *et al.* (*Tetrahedron Letters*, 22:1859-1862, 1981). One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Patent No. 4,458,066.

Micronuclei nucleic acid amplified in accordance with the invention contains a specific known nucleic acid sequence or an unknown target nucleic acid. Thus, the nucleic acid starting materials that can be employed include, for example, DNA or RNA, including messenger RNA, wherein DNA or RNA may be single stranded or double stranded. If RNA is to be used as a template, enzymes, and/or conditions optimal for reverse transcribing the template to DNA are utilized. In addition, a DNA-RNA hybrid which contains one strand of each may also be utilized. Moreover, a mixture of nucleic acids may also be employed, or nucleic acids produced in a previous amplification reaction using the same or different primers may be utilized. The nucleic acid sequence to be amplified, may be a portion of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor fraction of a complex mixture, such as contained in whole human DNA.

The agent for polymerization in the amplification reaction may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Taq polymerase, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, polymerase muteins, reverse transcriptase, ligase, and other enzymes, including heat-stable enzymes (*i.e.*, those enzymes which perform primer extension after being subjected to temperatures sufficiently elevated to cause denaturation). Suitable enzymes will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each template strand of target nucleic acid. Generally, the amplification will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths. There may be agents for amplification, however, which initiate synthesis at the 5' end and proceed in the other direction, using the same process as described above. In any event, the method of the invention is not limited to the embodiments of amplification which are described herein.

The amplified product may be detected by analyzing it by Southern blots without using radioactive probes. In such a process, for example, a small sample of nucleic acid containing a very low level of target nucleotide sequence is amplified, and analyzed via a Southern blotting technique. The use of non-radioactive probes or labels is facilitated by the high level of the amplified signal. For determination of the identity of the isolated amplified target nucleic acid, probes which hybridize to known amplified sequences may be used first to positively identify the target sequence or to eliminate the possibility that the amplified sequence is a previously identified gene (*e.g.*, myc, neu, PRAD, MDR1).

Nucleic acids having an amplified target sequence detected in the method of the invention can be further evaluated, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the analysis of a specific DNA sequence such as PCR, oligomer restriction (Saiki, *et al.*, *Bio/Technology*, 3:1008-1012, 1985), allele-specific oligonucleotide (ASO) probe analysis (Conner, *et al.*, *Proc. Natl. Acad. Sci. USA*, 80:278, 1983), oligonucleotide ligation assays (OLAs) (Landegren, *et al.*, *Science*, 241:1077, 1988), fluorescent in situ hybridization (FISH) and the like. Molecular techniques for DNA analysis have been reviewed (Landegren, *et al.*, *Science*, 242:229-237, 1988).

DNA sequences amplified by any one of a variety of means, can be cloned by several methods. For example, the DNA can be isolated using hybridization procedures which are well known in the art. These include, but are not limited to : 1) hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences; 2) antibody screening of expression libraries to detect shared structural features and 3) synthesis by the polymerase chain reaction (PCR).

Hybridization procedures are useful for the screening of recombinant clones by using labeled mixed synthetic oligonucleotide probes where each probe is potentially the complete complement of a specific nucleic acid sequence in the hybridization sample which includes a heterogeneous mixture of denatured double-stranded nucleic acid. For

such screening, hybridization is preferably performed on either single-stranded nucleic acid or denatured double-stranded nucleic acid. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al., Nucleic Acid Research*, 9:879, 1981).

The analysis of specific DNA sequences encoding amplified target nucleic acid sequences can also be obtained by: 1) isolation of double-stranded DNA sequences from the genomic DNA; 2) chemical synthesis of a DNA sequence to provide the necessary codons for the polypeptide of interest; and 3) *in vitro* synthesis of a double-stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double-stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA. Of these three methods for developing specific DNA sequences for use in recombinant procedures, the isolation of genomic DNA isolates is the least common. This is especially true when it is desirable to obtain the microbial expression of mammalian polypeptides due to the presence of introns.

Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid- or phage-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay *et al., Nucl. Acid Res.* 11:2325, 1983). One method of isolating cDNA

sequences representative of the amplified target nucleic acid in the micronuclei also includes a subtractive library approach for isolating cDNA clones derived from mRNAs exhibiting higher or lower abundance.

A cDNA expression library, such as lambda gt11, can be screened indirectly for the expression product of the amplified target nucleic acid by identifying a polypeptide having at least one epitope, using antibodies specific for Amplified target nucleic acid. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of amplified target nucleic acid cDNA.

An amino acid sequence of a polypeptide can be deduced from a target nucleic acid utilizing the genetic code, however, the degeneracy of the code must be taken into account. Polynucleotides of the invention include sequences which are degenerate as a result of the genetic code. The polynucleotides of the invention include sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, as long as the amino acid sequence of an amplified target nucleic acid results in a functional polypeptide (at least, in the case of the sense polynucleotide strand), all degenerate nucleotide sequences are included in the invention.

There are other methods of amplification and cloning of the amplified target nucleic acid of the invention including microdissection-PCR approach which can generate PCR products for use as probes to identify phage, cosmid or YAC clones in existing libraries of normal human DNA. RNA arbitrarily primed PCR, "RAP" or "differential display" can be used to produce a DNA fingerprint of the transcribed RNA that is suitable for molecular cloning (Welsh, *et al, Nucl. Acids Res.*, 20:4965, 1992; Liang, *et al., Science*, 257:967, 1992; Wong, *et al., Int. J. Oncol.*, 3:3, 1993, all incorporated by reference, herein).

The amplified target nucleic acid of the invention is a gene which encodes a protein such as a transcriptional regulator, a growth factor receptor, an inhibitor of the cell cycle, a G-protein, and a cell cycle-associated gene. Examples of such genes include, but are not limited to *c-myc*, *N-myc*, *Her-2/neu*, *PRAD1*, *erbB-2*, and *H-ras*. The amplified target nucleic acid may also be a gene which is referred to as a drug-resistance gene. Amplification of such genes, including dihydrofolate reductase (DHFR), carbamyl-phosphate synthetase-aspartate transcarbamylase-dihydroorotase (CAD) and multidrug resistance gene-1 (MDR1), provide a mechanism for a cell to avoid cell death upon treatment with the appropriate drug.

The method of the invention allows isolation of an amplified target nucleic acid which may be a previously known or an unknown gene. One of skill in the art will be able to use an amplification method, such as PCR, to isolate the gene for further identification. Probes which identify known genes are known and available to those of skill in the art. By elimination, one can readily determine if an amplified nucleic acid is a previously identified gene or not.

A cell proliferative disorder may be for example, associated with increased transcription and translation of an amplified target DNA sequence. The term "cell-proliferative disorder" denotes malignant as well as non-malignant cell populations which morphologically often appear to differ from the surrounding tissue. For example, the method of the invention may be useful in diagnosing malignancies of the various organ systems, such as, for example, lung, breast, lymphoid, hematopoietic, gastrointestinal, and genito-urinary tract as well as adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer, non-small cell carcinoma of the lung, ovarian cancer, brain cancer, uterine cancer, bladder cancer, cancer of the small intestine, and cancer of the esophagus.

The method is also useful in diagnosing non-malignant or immunological-related cell-proliferative diseases such as psoriasis, pemphigus vulgaris, Behcet's syndrome, acute

respiratory distress syndrome (ARDS), ischemic heart disease, post-dialysis syndrome, leukemia, rheumatoid arthritis, acquired immune deficiency syndrome, vasculitis, lipid histiocytosis, septic shock and inflammation in general. Essentially, any disorder which is etiologically linked to amplified nucleic acid target sequence would be considered a cell proliferative disorder as described herein.

The materials for use in the method of the invention are ideally suited for a kit. Such a kit comprises an agent which induces micronuclei formation, and means for amplifying target nucleic acid in the micronuclei, wherein the means comprises the necessary enzyme(s) and oligonucleotides for amplifying the target nucleic acid from a cell suspected of having a proliferative disorder.

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

EXAMPLES

Various cell lines that are known in the art to contain amplified nucleic acid sequences on extrachromosomal DMs, as well as cells suspected of having amplified nucleic acid sequences can be utilized in the method of the invention. The following illustrative examples utilized cell lines which are known to contain DMs having amplified oncogene sequences.

EXAMPLE 1

ANALYSIS OF CELL LINES WITH DMs

Cell Lines

An early passage (passage 46) of the HL60 promyelocytic leukemia cell line was obtained from S. Colins (Fred Hutchinson Cancer Center). This cell line contains 16-32 copies of the *MYC* oncogene, the majority of which localize to extrachromosomal molecules ranging from 250-kbp episomes to DMs (Collins, *et al., Nature,* 270:347-349, 1977; and Von Hoff, *et al., Proc. Natl. Acad. Sci., USA,* 85:4804-4808, 1988). Passage 67 subclone 173 contains a median of 8 DMs per cell and was used for this study (Van Hoff, *et al., supra*; and Von Hoff, *et al., J. Clin. Invest.,* 85:1887-1895, 1990). A previously described subclone of COLO 320DM (American Type Culture Collection; Quinn, *et al., Cancer Res.,* 39:4914-4924, 1979), which contains a median of 30 DMs per cell and an amplicon of 120-160 kbp (Von Hoff, *et al., supra*), was used. The COLO 320 HSR (HSR, homogeneously staining region) line has approximately the same number of copies of *MYC* dispersed at several chromosomal sites (Von Hoff, *et al., supra*). The NB4 neuroblastoma cell line (passage 20; kindly provided by J. Casper and V. Piaskowski, Milwaukee Children Hospital) has an ≈50-fold amplification of the *NMYC* gene localizing to ≈1000-kbp episomes and DMs (VanDevanter, *et al., J. Natl. Cancer Inst.,* 82:1815-1821, 1990). The SF188 glioblastoma multiform cell line, passage 220, has a 25-fold amplification of the *MYC* gene localizing to heterogeneously sized episomes, with a minimum size of 100 kbp, and DMs (Trent, *et al., Proc. Natl. Acad. Sci, USA,* 83:470-473, 1986).

The HL60 and COLO 320 cell lines were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), and NB4 and SF188 in RPMI 1640 medium containing 20% FBS and 2 mM glutamine. Based on previous work by Snapka and Varshavsky, *Proc. Natl. Acad. Sci., USA,* 807533-7537, 1983, HU (Squibb) was added on day 0 of culture at the concentrations indicated and was replaced each time the cells were passaged. All cells were passaged by a 1:10 dilution of confluent cultures every 3-7 days. Cell growth was determined with a hemocytometer.

Evaluation of Cells for Micronuclei and Localization of *MYC* Genes

Micronuclei were scored in preparations of metaphase chromosome spreads and interphase nuclei (Von Hoff, *et al., supra*; and Naylor, *et al., Methods Enzymol.,* 151:279-292, 1987). The cells were exposed to Colcemid (0.1 μg/ml; GIBCO) for 1-3 hours, incubated in 0.075 M KCl for 20 minutes, fixed in methanol/glacial acetic acid (3:1), and dropped on wet slides. Portions of tumors that had been established *in vivo* were either used immediately for preparation of metaphase spreads or reestablished in cell culture to enable a comparison of the number of *MYC* DMs per cell under various growth conditions.

The *MYC* cosmid (Yuxin Yin, Salk Institute) and centromere probes (Oncor, Gaithersburg, MD) used for *in situ* hybridization were labeled with biotin-16-dUTP (Boehringer Mannheim) by nick-translation with a reaction mixture containing all four dNTPs (Pharmacia). Fluorescent *in situ* hybridization (FISH) was conducted as described by Pinkel, *et al., (Proc. Natl. Acad. Sci., USA,* 83:2934-2938, 1986).

Exposure of Cells to Hydroxyurea

All cells were continuously exposed to concentrations of hydroxyurea (Sigma) of 50, 100, and 200 μM.

EXAMPLE 2

PURIFICATION OF MICRONUCLEI FROM CELLS CONTAINING AMPLIFIED GENES AS DM AND/OR EPISOMES

Cell lines

Human Colo320DM or Colo320HSR neuroendocrine tumor cells (Alitalo, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 1707-1711 (1983)) were provided by D.D. Vonhoff (University of Texas, San Antonio) and grown in RPMI1640 medium supplemented with 10% fetal calf serum. When the cell density reached $1.5x10^6$ cells/ml, a subculture was made by diluting to $2.5x10^5$ cells/ml with growth medium. The locations of amplified c-*myc* genes to DMs or HSRs were confirmed by FISH using c-*myc* cosmid DNA (from Yuxin Yin, Salk Institute). B-1/50 cell line (Yeung, *et al.*, *J. Biol Chem.*, 258, 8338-8345 (1983); Nonet, *et al.*, *Genomics* 15, 543-558 (1993)) was provided by R. Kellems (Texas Medical Center) and grown in DMEM supplemented with 1x MEM nonessential amino acids (GIBCO BRL), 15% heat-activated horse serum, 50 μM 2'-deoxycofomycin, 1.1 mM adenosine, 20 μM azaserine, and 1 mM uridine. 2'-Deoxycofomycin was obtained from the National Cancer Institute. XEW8.2.3 cell line (Carine, *et al.*, *Somat. Cell Mol. Genet.*, 12, 479-491 (1986; Carine, *etal.*, *Somat. Cell Mol. Genet.* 15, 445-460 (1989)) was developed by and provided from I. Scheffler (University of California, San Diego) and maintained in DMEM supplemented with 10% fetal calf serum. Human diploid WS-1 cell line was obtained from American Type Culture Collection (CRL 1502) (ATCC, Rockville, MD) and maintained in DMEM supplemented with 10% heat activated fetal calf serum and 1 x MEM nonessential amino acids. All cells were grown at 37 °C with 7% CO_2. Hydroxyurea (Sigma) of indicated final concentration was added to the culture when it was subcultured to lower cell density. Cells were grown in the presence of hydroxyurea for, unless otherwise noted, 3 days.

Purification of micronuclei

This procedure was developed, in some portion, based on the protocol for the isolation of intact nuclei (Janssen, K. *et al., Current Protocols in Molecular Biology*, p4.10.1-4.10.11, John Wiley & Sons, Inc., NY). FIGURE 1 shows a flow chart of the isolation protocol used for isolation of micronuclei and amplified nucleic acid sequences contained within the micronuclei. The cells (~ $1x10^9$ cells) were treated with 100 μM hydroxyurea for 3 days, harvested and washed twice with DMEM without serum by centrifuging 200 x g for 5 min. The cell precipitate was resuspended in 20 ml of prewarmed DMEM containing cytochalasin B (10 μg/ml) and incubated for 30 min at 37 °C. After collection at 200 x g for 5 min, cells were resuspended in 10 ml of prechilled lysis buffer (10 mM Tris-Cl, 2 mM Mg-acetate, 3 mM $CaCl_2$, 0.32 M sucrose, 0.1 mM EDTA, 1 mM Dithiothreitol, 0.5% (v/v) Nonidet P-40, 0.15 mM spermine, 0.75 mM spermidine and 10 μg/ml cytochalasin B, pH 8.5), and Dounce homogenized (5 to 10 strokes using loose fitting pestle). The release of micronuclei from cytoplasm or nuclei was confirmed by mixing small portion of homogenate with equal volume of PBS containing 2 μg/ml of DAPI and 0.1 μg/ml of PI, and examining under fluorescence microscope using triple band path filter. Then, the homogenate was combined with the equal volume of 2M sucrose buffer (10 mM Tris-Cl, 2M sucrose , 5 mM Mg-acetate, 0.1 mM EDTA, 1mM dithiothreitol, pH 8.0, 4 °C), and was layered onto the top of sucrose stepwise gradient (10, 5, and 5 ml of sucrose buffer containing 2 M, 1.8 M, and 1.6 M of sucrose respectively from the bottom of tube), and centrifuged in SW28 rotor, 14,000 rpm, 45 min at 4 °C. After centrifugation, the pellet and the interphase between 2 and 1.8M sucrose layer, which contain nuclei and micronuclei, were recovered, and washed twice by PBS- by centrifuging 1,000 x g, 20 min at 4 °C. The suspension (3ml) was then layered on the top of linear BSA gradient (0.5 to 4% in PBS, total volume 40 ml, made in 50 ml disposable syringe) (Dhar, *et al., Somat. Cell Mol. Genet.*, 10:547-559, 1984), and allowed to sit at 4 °C for 4 hours. Fractions (2ml each) were taken from the top of gradient, diluted by PBS and centrifuged at 1,000 x g for 20 min. The precipitate from fractions 1 to 8 were suspended in PBS, mixed and re-fractioned by the BSA gradient sedimentation as above. Small portion of each fraction was stained with DAPI and

examined under fluorescence microscope. About 90% of DAPI positive particles present in fractions 1 to 4 were shown to typical micronuclei based on the size, the shape and the property of DAPI staining. Fractions 1 to 4 were pooled and treated with DNase I(5 μg/ml) and RNase A (40 μg/ml) for 30 min at 37 °C. This fraction was determined to be purified micronuclei thereafter. A portion of purified micronuclei was fixed by methanol/ acetic acid (3/1), and examined by *in situ* hybrization using c-*myc* cosmid probe. Remaining samples were treated with proteinase K (60 μg/ml) and 0.05% triton X-100 for 60 min at 50% followed by the inactivation of the enzyme at 94 °C for 12 min, and used for the gene quantitation by PCR or the generation of FISH probe.

Gene quantitation by PCR

The amount of c-*myc* gene amplified on DMs in Colo320DM cells was quantitated and control β-globin gene, single copy on chromosome 11, by competitive PCR procedure as described (Siebert, *et al.*, *BioTechniques*, 14, 244-249, 1993; Forster, *et al.*, *BioTechniques*, 16, 1006-1008, 1994). The sequences of primers used for c-*myc* gene were myc-C, 5'd(CTG GGA TCT TCT CAG CCT AT)3' (SEQ ID NO:1) and myc-D, 5'd(ACT CCT CTC ACC ATG AAG GT)3' (SEQ ID NO:2). The sequences of primers used for β-globin were IVS-I, 5'd (GTA TCA TGC CTC TTT GCA CC)3' (SEQ ID NO:3), and IVS-L, 5'd(AAG GGC CTA GCT TGG ACT CA) (SEQ ID NO:4). The primer set of myc-C and myc-D amplifies 400 bp product from human c-*myc* gene intron 2, and the primer set of IVS-I and IVS-L amplifies 214 bp product form human β-globin gene intron 2. Internal standards for each genes were prepared by PCR amplification using c-*myc* or β-globin primer pairs from salmon DNA or *Saccharomyces pombe* DNA, respectively. At that time, annealing temperature was lowered to 42 C or 47°C for c-*myc* and β-globin, respectively. The products were separated by agarose gel electrophoresis, and the bands of ca. 200bp or ca. 400 bp for c-*myc* or β-globin, respectively, were excised. Standard DNAs in the excised bands were further purified by successive 3 rounds of PCR amplification at higher annealing temperature (63 °C) and fractionation by agarose gel electrophoresis. The amount of final PCR products, each which gave single band in agarose gel electrophoresis, were quantitated by the intensities of the

ethidium bromide-stained bands in agarose gel, and used as the standard DNA for the quantitation of test DNA.

For this purpose, a series of PCR reactions were made containing equal amount of test DNA and serial 2-fold diluted standard DNA. Each tube (10μl) contain 1 x Taq buffer (Invitrogen; N for c-*myc*, J for β-globin), 0.2 mM each of dNTP, 20 ng each of primers, 0.2 μl of test DNA, serially diluted known amount of standard DNA, and 0.4 u of Taq polymerase (Boehringer Manheim). The tubes were heated to 95 °C for 3 min followed by 40 cycles at 94 °C for 1 min, 63 °C for 1 min, 72 °C for 2 min. After the end of PCR, the products were seperated by agarose gel electphoresis, stained with ethidium bromide and the intensities of the products from test and standard DNA, differing in the size, were compared and evaluated.

The generation of FISH probe from purified micronuclei

DNA in the proteinase K-treated micronuclei preparation was uniformly amplified by randomly primed PCR as reported by Telenius, *et al.* (Telenius, *et al., Genes Chrom. Cancer,* 4, 257-263, 1992) Briefly, an initial 8 cycles of PCR (94 °C for 1 min, 30 °C for 4 min, 37 °C for 2.5 min) was conducted in 5 μl of 1 x Sequenase reaction buffer (USB), 0.2 mM each dNTP, and 10 μM of Telenius primer by adding 0.2 u of Sequenase (Ver. 2.0 USB) at each cycle. After this step, a conventional PCR was performed in the same tube by adding 50 μl of reaction mixture containing 1 x Taq buffer B (Invitrogen), 0.2 mM each of dNTP, 2 μM of Telenius primer, 2.5 u of Taq DNA polymerase (Boehringer Mannheim). The reaction was heated to 95 °C for 3 min followed by 35 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min. Amplified products were FITC-labeled by "Prime-it Fluor Fluorescence labeling kit" (Stratagene) primed with Telenius primer instead with random hexanucleotides supplied in the kit. In some experiments, uniformly amplified products were labeled by biotin using "BioPrime DNA System" (Life Technologies) as per the manufacturer's protocol.

FISH

Metaphase spreads were prepared as described and treated with RNase (100 µg/ml in 2 x SSC, 37 °C, 60 min.) Hybridization of the FISH probes was as described (Pinkel, *et al.*, *Proc. Natl. Acad. Sci., USA,* 83:2934-2938, 1986). Briefly, for each hybridization, 50 ~100 ng of probe was used in 15 µl hybridization mixture (containing 50% formamide, 10% dextran sulfate, 2x SSC, 6 µg salmon sperm DNA, and 3 µg human or mouse COT I DNA (BRL) depending on the probe species used) which was denatured at 75 °C for 5 min followed by 42 °C, 30 min. The slides with metaphase spreads was denatured in 70% formamide, 2x SSC at 70~72 °C for 2 min, rinsed in ice cold 70%, 85% and 100% ethanol for 3 min each, and air dried. The hybridization with probes was done at 37 °C in a moist chamber overnight. The slide was then washed three times in 50% formamide, 2x SSC at 45 °C for 3 min each, three times in 2xSSC at 45°C for 3 min each, and one time in 0.1 x SSC at 60 °C for 10 min. The slide was viewed at this point when FITC labeled probes were used. Alternatively, the hybridization signal of biotin labeled probe was detected with one layer of FITC-conjugated avidin (Vector) and amplified with biotinylated anti-avidin (Vector) and a second layer of FITC-conjugated avidin. The slide was counter stained with 0.5 µg/ml of propidium iodide in Vectashield (Vector) and was examined with a Zeiss fluorescence microscope equipped with appropriate epifluorescence filters.

The results of the FISH studies confirmed the isolation of micronuclei and DMs by the method described herein. FIGURE 2a shows the frequency of micronuclei having c-myc versus the total number of interphase nuclei in COLO 320DM cells. FIGURE 2b shows the frequency of micronuclei in interphase nuclei in B-1/50 cells (mouse cells having amplification of adenosine deaminase gene). FIGURE 2c shows the frequency of micronuclei in interphase nuclei in XEW 8.2.3 cells (CHO cells having human centromere sequences and minichromosome). FIGURE 3 shows DNA in purified micronuclei from COLO 320DM cells (FITC labeled) and FISH hybridized to COLO 320DM metaphase spreads (x 1000 magnification).

FIGURE 4 shows micronuclei formed in COLO 320DM cells and FISH hybridized to c-myc cosmid DNA and detected by FITC (x1000 magnification). FIGURE 5 shows purified micronuclei from COLO 320DM cells treated with HU (100 uM) for 3 days and FISH hybridized to c-myc cosmid DNA (x400 magnification).

Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

CLAIMS

1. A method for isolating an amplified target nucleic acid associated with a cell proliferative disorder comprising:

subjecting a cell suspected of having a cell proliferative disorder to conditions sufficient to produce micronuclei;

isolating the micronuclei from the cell;

amplifying target nucleic acid in the micronuclei, wherein the target nucleic acid is associated with a cell proliferative disorder; and

isolating the amplified target nucleic acid.

2. A method for isolating an amplified target nucleic acid associated with a cell proliferative disorder comprising:

isolating the amplified target nucleic acid wherein said target nucleic acid is associated with a cell proliferative disorder and is located in micronuclei isolated from a cell(s) subjected to conditions sufficient to produce micronuclei.

3. A method for isolating an amplified target nucleic acid associated with a cell proliferative disorder comprising:

amplifying target nucleic acid in micronuclei isolated from cells subjected to conditions sufficient to produce micronuclei, wherein said target nucleic acid is associated with a cell proliferative disorder; and

isolating the amplified target nucleic acid.

4. A method for isolating an amplified target nucleic acid associated with a cell proliferative disorder comprising:

isolating micronuclei from a cell subjected to conditions sufficient to produce micronuclei;

amplifying target nucleic acid in the micronuclei wherein the target is associated with a cell proliferative disorder; and

isolating the amplified target nucleic acid.

5. The method of claim 1, wherein the cell is subjected to an agent which induces the formation of micronuclei in a cell.

6. The method of claim 5, wherein the agent is selected from the group consisting of an inhibitor of DNA replication, a DNA damaging agent, an inhibitor of topoisomerase II, and a membrane disrupting agent.

7. The method of claim 6, wherein the agent is hydroxyurea.

8. The method of claim 1, wherein isolation of micronuclei is accomplished by physical separation, density gradient separation and/or immunoseparation.

9. The method of claim 8, wherein physical separation is differential centrifugation.

10. The method of claim 8, wherein immunoseparation is by means of an antibody having the specificity of an anti-nuclear pore antibody.

11. The method of claim 1, wherein amplifying is accomplished by polymerase chain reaction.

12. The method of claim 1, wherein the target nucleic acid is a gene which encodes a protein selected from the group consisting of a transcriptional regulator, a growth factor receptor, an inhibitor of the cell cycle, a G-protein, and a cell cycle-associated gene.

13. The method of claim 12, wherein the target nucleic acid is a gene selected from the group consisting of c-myc, N-myc, Her-2/neu, PRAD1, erbB-2, and H-ras.

14. The method of claim 1, wherein the target nucleic acid is a drug-resistance gene.

15. The method of claim 14, wherein the drug resistance gene is selected from the group consisting of dihydrofolate reductase (DHFR), carbamylphosphate synthetase-aspartate transcarbamylase-dihydroorotase (CAD) and multidrug resistance gene-1 (MDR1).

16. The method of claim 1, wherein the cell is derived from a tissue selected from lung, breast, colon, ovary, blood, brain, bladder, and uterus.

17. A kit useful for the isolation of target nucleic acid associated with a cell proliferative disorder, the kit comprising an agent which induces micronuclei formation, and means for amplifying target nucleic acid in the micronuclei, said means comprising the necessary enzyme(s) and oligonucleotides for amplifying said target nucleic acid from a cell suspected of having a proliferative disorder.

18. The kit of claim 17, wherein the agent is selected from the group consisting of an inhibitor of DNA replication, a DNA damaging agent, an inhibitor of topoisomerase II, and a membrane disrupting agent.

19. The kit of claim 18, wherein the agent is hydroxyurea.

20. The kit of claim 17, further comprising a density gradient separation medium.

21. The kit of claim 17, further comprising an antibody having the specificity of an anti-nuclear pore antibody.

22. The kit of claim 17, wherein the target nucleic acid is a gene which encodes a protein selected from the group consisting of a transcriptional regulator, a growth factor receptor, an inhibitor of the cell cycle, a G-protein, and a cell cycle-associated gene.

23. The kit of claim 17, wherein the target nucleic acid is a gene selected from the group consisting of c-myc, N-myc, Her-2/neu, PRAD1, erbB-2, and H-ras.

24. The kit of claim 17, wherein the target nucleic acid is a drug-resistance gene.

25. The kit of claim 24, wherein the drug resistance gene is selected from the group consisting of dihydrofolate reductase (DHFR), carbamylphosphate synthetase-aspartate transcarbamylase-dihydroorotase (CAD) and multidrug resistance gene-1 (MDR1).

26. The kit of claim 17, wherein the cell is derived from a tissue selected from lung, breast, colon, ovary, blood, brain, bladder, and uterus.

ABSTRACT

The present invention provides a method for the isolation of extrachromosomal amplified nucleic acids that are associated with a cell proliferative disorder. Isolation and further identification of such genes is critical for diagnosis, prognosis, and course of therapy.

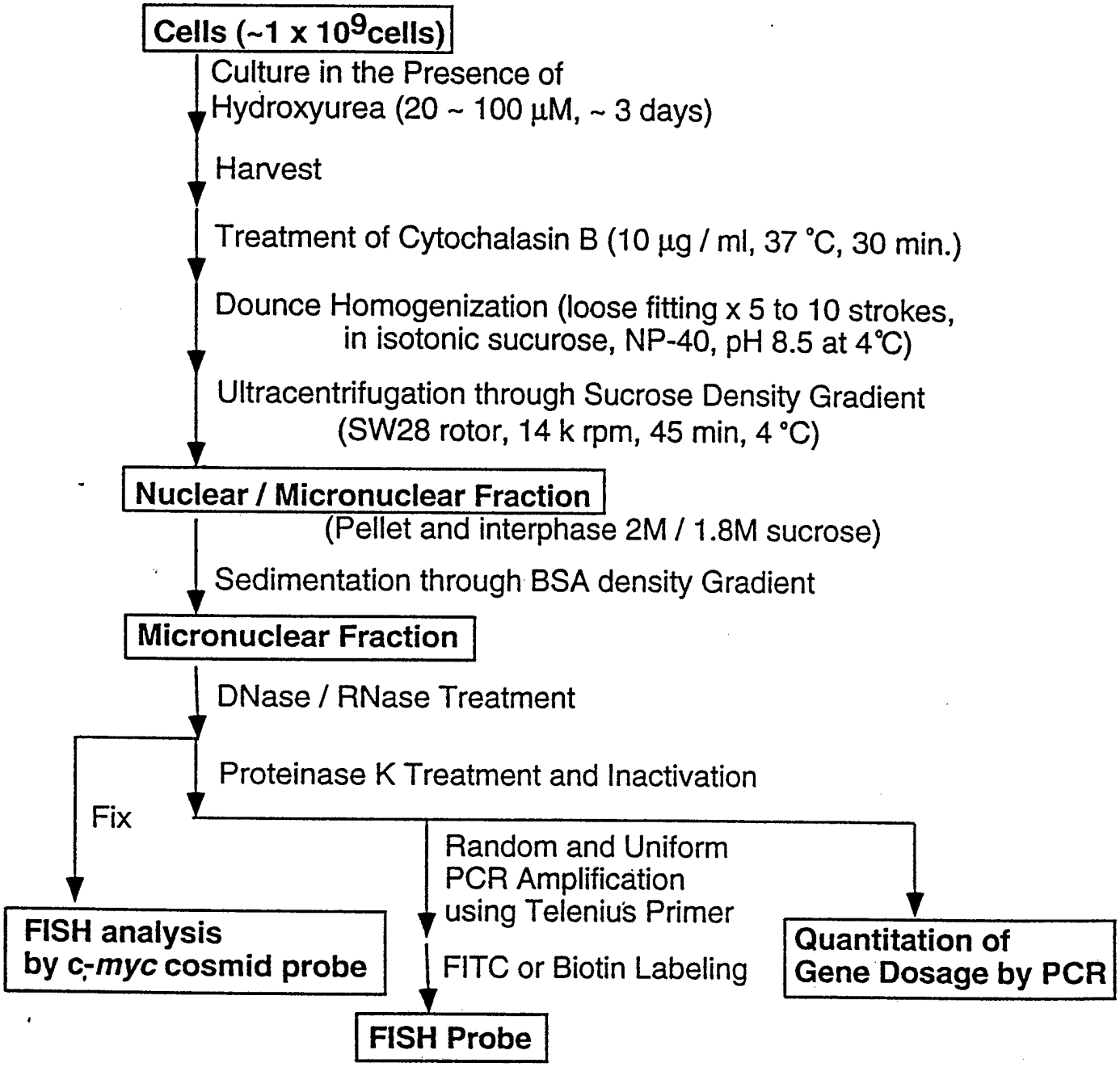

Cells (~1 x 10^9cells)
Culture in the Presence of
Hydroxyurea (20 ~ 100 µM, ~ 3 days)
Harvest
Treatment of Cytochalasin B (10 µg / ml, 37 °C, 30 min.)
Dounce Homogenization (loose fitting x 5 to 10 strokes,
in isotonic sucurose, NP-40, pH 8.5 at 4°C)
Ultracentrifugation through Sucrose Density Gradient
(SW28 rotor, 14 k rpm, 45 min, 4 °C)
Nuclear / Micronuclear Fraction
(Pellet and interphase 2M / 1.8M sucrose)
Sedimentation through BSA density Gradient
Micronuclear Fraction
DNase / RNase Treatment
Proteinase K Treatment and Inactivation
Fix
Random and Uniform
PCR Amplification
using Telenius Primer
FITC or Biotin Labeling
FISH analysis
by c-myc cosmid probe
FISH Probe
Quantitation of
Gene Dosage by PCR

FIGURE 1

FIGURE 2a

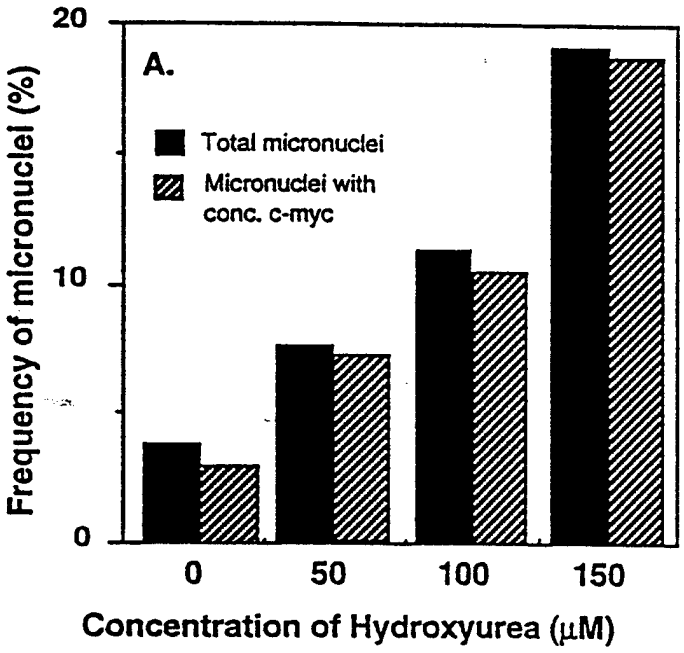

20
10
0
A.
Total micronuclei
Micronuclei with conc. c-myc
Frequency of micronuclei (%)
0
50
100
150
Concentration of Hydroxyurea (μM)

FIGURE 2b

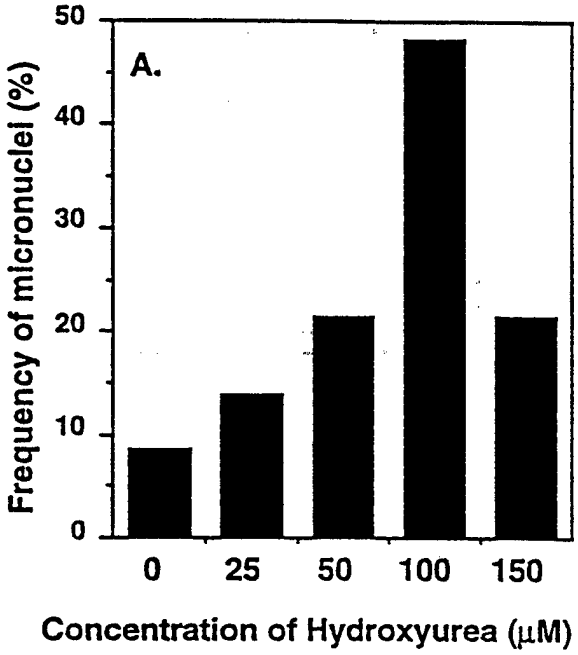

A.
Frequency of micronuclei (%)
50
40
30
20
10
0
0
25
50
100
150
Concentration of Hydroxyurea (μM)

FIGURE 2c

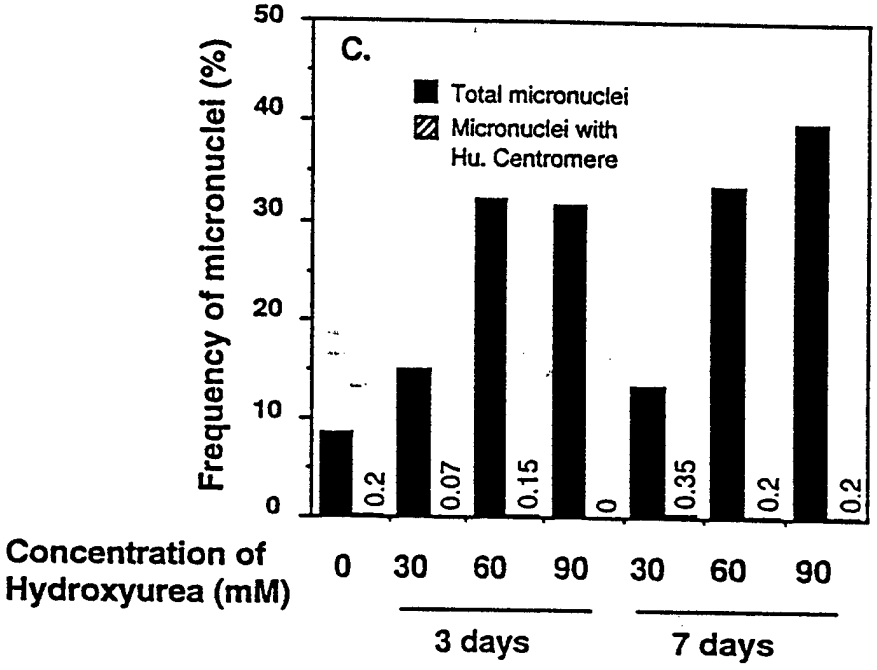

C.
Total micronuclei
Micronuclei with Hu. Centromere
Frequency of micronuclei (%)
50
40
30
20
10
0
0.2
0.07
0.15
0
0.35
0.2
0.2
Concentration of Hydroxyurea (mM)
0
30
60
90
30
60
90
3 days
7 days

FIGURE 3

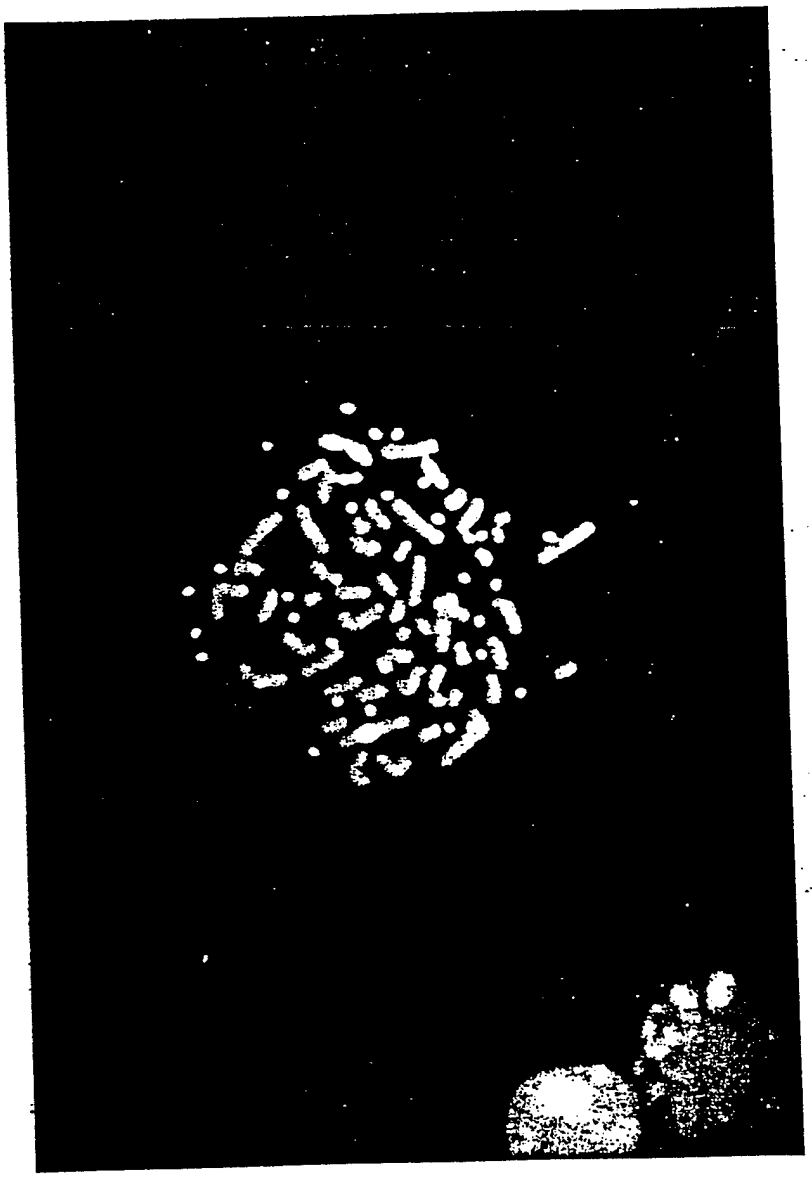


FIGURE 4

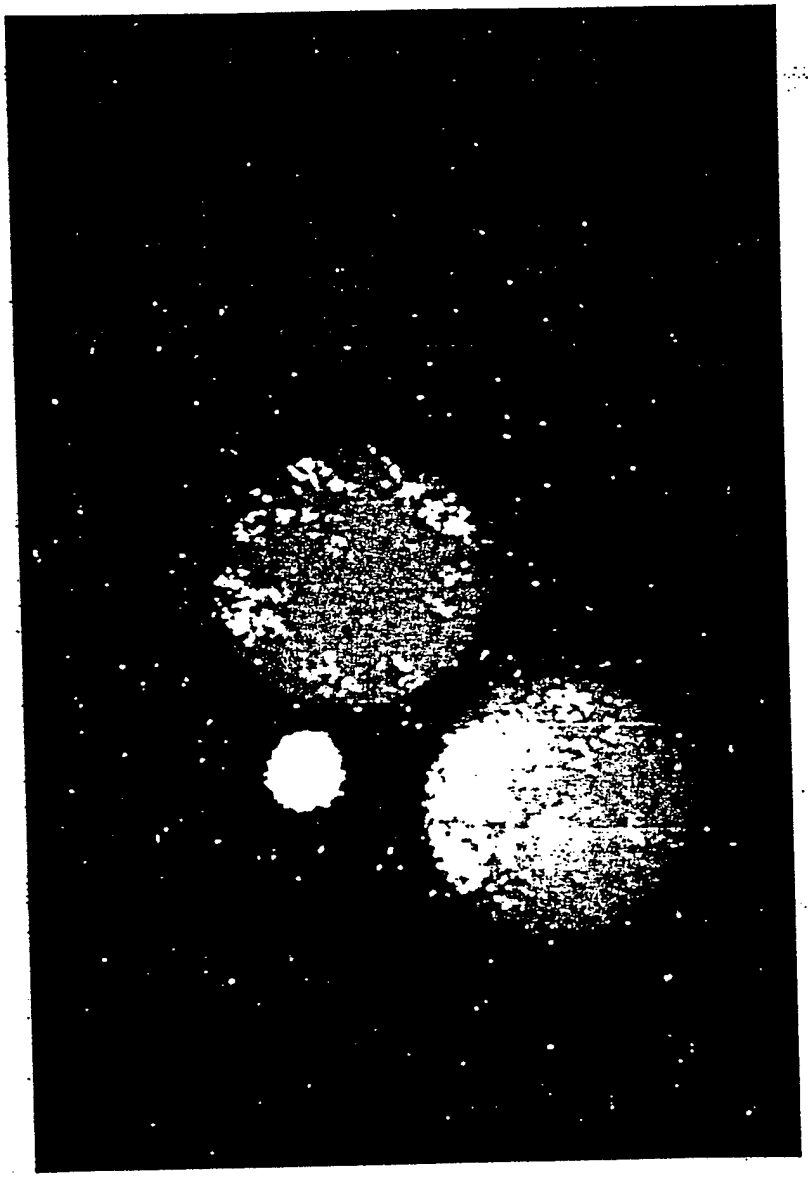
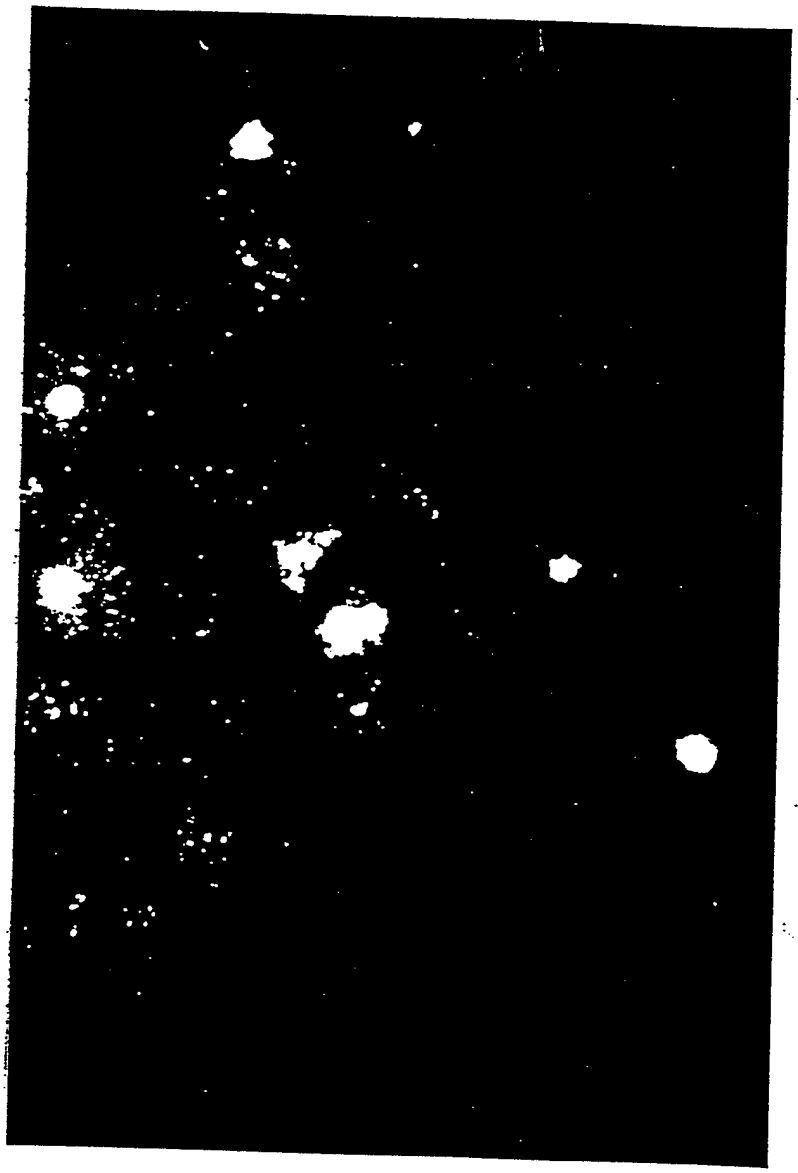


FIGURE 5



PENDING CLAIMS FOR USSN 08/704,391 FILED AUGUST 26, 1996:

1. A method for isolating an extrachromosomal amplified target nucleic acid in a cell comprising:contacting a cell suspected of having extrachromosomal amplified target nucleic acid with a non-alkaloid agent capable of inducing the formation of micronuclei in the cell, under conditions sufficient to produce micronuclei;
isolating the micronuclei from the cell;
amplifying target nucleic acid in the micronuclei; and
isolating the amplified target nucleic acid.

6. The method of claim 1, wherein the agent is selected from the group consisting of an inhibitor of DNA replication, a DNA damaging agent, an inhibitor of topoisomerase II, and a membrane disrupting agent.

7. The method of claim 6, wherein the agent is hydroxyurea.

8 The method of claim 1, wherein isolation of micronuclei is accomplished by physical separation, density gradient separation and/or immunoseparation.

9. The method of claim 8, wherein physical separation is differential centrifugation.

10. The method of claim 8, wherein immunoseparation is by means of an antibody having the specificity of an anti-nuclear pore antibody.

11. The method of claim 1, wherein amplifying is accomplished by polymerase chain reaction.

12. The method of claim 1, wherein the target nucleic acid is a gene which encodes a protein selected from the group consisting of a transcriptional regulator, a growth factor receptor, an inhibitor of the cell cycle, a G-protein, and a cell cycle-associated gene.

13. The method of claim 12, wherein the target nucleic acid is a gene selected from the group consisting of c-myc, N-myc, Her-2/neu, PRAD1, erbB-2, and H-ras.

14. The method of claim 1, wherein the target nucleic acid is a drug-resistance gene.

15. The method of claim 14, wherein the drug resistance gene is selected from the group consisting of dihydrofolate reductase (DHFR), carbamylphosphate synthetase-aspartate transcarbarnylase-dihydroorotase (CAD) and multidrug resistance gene-1 (MDR1).

16. The method of claim 1, wherein the cell is derived from a tissue selected from lung, breast, colon, ovary, blood, brain, bladder, and uterus.

17. A kit useful for the isolation of an extrachromosomal target nucleic acid in a cell, the kit comprising a non-alkaloid agent which induces micronuclei formation, and means for amplifying target nucleic acid in the micronuclei, said means comprising the necessary enzyme(s) and oligonucleotide for amplifying said extrachromosomal target nucleic acid from the cell.

18. The kit of claim 17, wherein the agent is selected from the group consisting of an inhibitor of DNA replication, a DNA damaging agent, an inhibitor of topoisomerase II, and a membrane disrupting agent.

19. The kit of claim 18, wherein the agent is hydroxyurea.

20. The kit of claim 17, further comprising a density gradient separation medium.

21. The kit of claim 17, further comprising an antibody having the specificity of an anti-nuclear pore antibody.

22. The kit of claim 17, wherein the target nucleic acid is a gene which encodes a protein selected from the group consisting of a transcriptional regulator, a growth factor receptor, an inhibitor of the cell cycle, a G-protein, and a cell cycle-associated gene.

23. The kit of claim 17, wherein the target nucleic acid is a gene selected from the group consisting of c-myc, N-myc, Her-2/neu, PRAD I, erbB-2, and H-ras.

24. The kit of claim 17, wherein the target nucleic acid is a drug-resistance gene.

25. The kit of claim 24, wherein the drug resistance gene is selected from the group consisting of dihydrofolate reductase (DHFR), carbamylphosphate synthetase-aspartate transcarbamylase-dihydroorotase (CAD) and multidrug resistance gene-1 (MDRI).

26. The kit of claim 17, wherein the cell is derived from a tissue selected from lung, breast, colon, ovary, blood, brain, bladder, and uterus.

27. The method of claim 7, wherein the hydroxyurea is from about 1 μM to 200 μM.

S95008

U.S. SERIAL NO. 452,275

as filed 5/26/95

APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: METHOD FOR ISOLATION OF EXTRACHROMOSOMAL AMPLIFIED GENES

APPLICANT: GEOFFREY M. WAHL
NORIAKI SHIMIZU

"EXPRESS MAIL" Mailing Label Number EG190458829US

Date of Deposit May 26, 1995

I hereby certify under 37 CFR 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office To Addressee" with sufficient postage on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Lori Linderman

Lori Linderman

METHOD FOR ISOLATION OF EXTRACHROMOSOMAL AMPLIFIED GENES

This invention was made with Government support under Grant No. CA 48405, awarded by the National Institutes of Health and Grant No. DAMD17-94-J-4359, awarded by the U.S. Army Medical Research Acquisition Activity. The Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

1. *Field of the Invention*

This invention relates generally to gene amplification and specifically to a method for isolation of extrachromosomal amplified nucleic acid sequences.

2. *Description of Related Art*

Gene amplification in tumor cells results in the production of multiple copies of a genomic region. Amplification of oncogenes leads to the over expression of proteins participating in the transduction of growth-related signals and confers a growth advantage to tumor cells. Clinically, oncogene amplification is extremely common in human tumors and correlates with a poor prognosis for patients with ovarian cancer (HER-2/neu), breast cancer (c-myc, HER-2/neu), neuroblastoma (N-myc), or small cell lung cancer (c-myc) (Slamon, *et al.*, *Science* 235:177, 1987; Slamon, *et al.*, *Science*, 244:707, 1989; Seeger, *et al.*, *N. Engl. J. Med.*, 313:1111, 1985; Johnson, *et al.*, *J. Clin. Invest.*, 78:525, 1986). There is also evidence that amplification of drug resistant genes is associated with both *in vitro* and *in vivo* resistance of a patient's tumor to an antineoplastic agent (Schimke, R., *Cancer Res.*, 44:1735, 1984; Stark, G., *Cancer Surv.* 5:1, 1986; Trent, *et al.*, *J. Clin. Oncol.*, 2:8, 1984; Curt, *et al.*, *N. Engl. J. Med.*, 308:199, 1983).

Amplified genes have been localized to two types of cytogenetically distinguishable structures. These structures can be located on the chromosome, within homogeneously staining regions (HSRs), or they can be reside extrachromosomal either as submicroscopic elements called episomes or as larger structures called double minute chromosomes (Carroll, *et al., Mol. Cell. Biol.*, 8:1525, 1988; Von Hoff, *et al., J. Clin. Invest.*, 85:1887, 1990; Von Hoff, *et al., Proc. Natl. Acad. Sci. USA*, 85:4804, 1988). The occurrence of DMs in a malignant cell line was first described by Spriggs, *et al.* (*Br Med J*, 2:1431, 1962). DMs are paired, acentric fragments that segregate randomly at cell division and can be detected in the majority of primary tumors at biopsy (Benner, *et al., Anticancer Drugs*, 2:11, 1991; Gebhart, *et al., Int. J. Cancer*, 34:369, 1984). DMs tend to vary in size and also in number of DMs per cell.

Genes amplified on DMs can be lost spontaneously at each cell division or can be eliminated by treatment with hydroxyurea (HU) at concentrations that do not inhibit DNA synthesis or ribonucleotide reductase (Von Hoff, *et al., Proc. Natl Acad Sci, USA*, 89:8165, 1992; Von Hoff, *et al., Cancer Res.*, 51:6273, 1991). It appears that HU-treatment of cells containing DMs results in an increase in micronuclei formation, and the capture of the DMs within the micronuclei (Von Hoff, *et al., Proc. Natl Acad Sci, USA*, 89:8165, 1992; Von Hoff, *et al., Proc. Am Assoc. Cancer Res.*, 33:359, 1992). By contrast, HSRs are not lost during cell division or by treatment with HU and represent a stable form of gene amplification. Elimination *c-myc* genes contained in DMs from a colon cancer cell line of neuroendocrine origin (COLO 320 DM) reduced its tumorigenicity in nude mice (Von Hoff, *et al., Proc. Natl. Acad. Sci., USA*, 89:8165, 1992). Studies have shown that treatment of HL60 cells with low concentrations of HU reduced the number of *c-myc*-containing DMs, which led to decreased *c-myc* expression and induction of differentiation (Eckhardt, *et al., Proc. Natl. Acad. Sci., USA*, 91:6674, 1994; Shimizu, *et al., Cancer Res.*, 54:3561, 1994). The studies also showed that agents which eliminate extrachromosomal DNA also alter tumor phenotype.

Similarly, previous studies have shown that when the selective pressure of a drug is removed from mammalian cells that carry unstably amplified genes on extrachromosomal particles, the cells gradually lose those amplified genes (lose their DMS or episomes). Snapka and Varshavsky previously showed that HU could increase the rate of loss of unstably amplified dihydrofolate reductase (DHFR) genes from mouse cells (*Proc. Natl. Acad. Sci., USA*, 80:7533, 1983). Von Hoff, *et al.*, showed a similar elimination of the multidrug resistance gene 1 (MDR1) gene in vinblastine resistant human squamous tumor cells, as well as carbamylphosphate synthetase, aspartate transcarbamylase, dihydro-orotase (CAD) genes from N-(phosphonacetyl)-L-aspartic acid (PALA) resistant Chinese hamster ovary cells, and DHFR genes from methotrexate resistant human squamous tumor cells (*Cancer Research*, 51:6273, 1991).

The persistence of DMs implies that these acentric elements express one or more genes that impart a growth or survival advantage to the cell. Identification of the expressed nucleic acid sequences contained in such DMs would provide a means for developing appropriate diagnostic, prognostic and therapeutic strategies.

SUMMARY OF THE INVENTION

The knowledge that gene amplification occurs in cancer cells provides an unparalleled opportunity for developing therapeutic approaches that are highly specific for tumor cells. The ability to eliminate amplified genes by removal or selective interference with their expression is enhanced by the determination of the identity of the amplified gene. The present invention provides a method for isolating and for identifying amplified genes which exist extrachromosomally in DMs within a cell.

The method of the invention allows isolation and molecular cloning of target nucleic acid sequences contained in extrachromosomal amplified loci. The identity of such nucleic acids or genes, whether previously known or unknown, provides a means for more accurate diagnosis and prognosis for a subject having a disorder such as a cancer. The identity of the genes also provides a method for monitoring the course of therapy for such a subject.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows a flow chart for isolation of micronuclei and amplified nucleic acid sequences contained within the micronuclei.

FIGURE 2a shows the frequency of micronuclei having c-myc versus the total number of interphase nuclei in COLO 320DM cells.

FIGURE 2b shows the frequency of micronuclei in interphase nuclei in B-1/50 cells (mouse cells having amplification of adenosine deaminase gene).

FIGURE 2c shows the frequency of micronuclei in interphase nuclei in XEW 8.2.3 cells (CHO cells having human centromere sequences and minichromosome).

FIGURE 3 shows DNA in purified micronuclei from COLO 320DM cells (FITC labeled) and FISH hybridized to COLO 320DM metaphase spreads (x 1000 magnification).

FIGURE 4 shows micronuclei formed in COLO 320DM cells and FISH hybridized to c-myc cosmid DNA and detected by FITC (x1000 magnification).

FIGURE 5 shows purified micronuclei from COLO 320DM cells treated with HU (100 uM) for 3 days and FISH hybridized to c-myc cosmid DNA (x400 magnification).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for isolating extrachromosomal amplified nucleic acid sequences from a cell having or suspected of having a cell proliferative disorder. Such disorders are associated with the amplification of cell growth control genes, oncogenes, multidrug resistance genes, and growth factor receptors, for example. The method of the invention provides a means for the identification of both previously known and of unknown expressed amplified nucleic acid sequences. Once the amplified nucleic acid is isolated and identified, probes can be developed for use in diagnosis, prognosis, and for monitoring a particular therapeutic regime.

In a preferred embodiment, the invention provides a method for isolating an amplified target nucleic acid associated with a cell proliferative disorder comprising subjecting a cell suspected of having a cell proliferative disorder to conditions sufficient to produce micronuclei, isolating the micronuclei from the cell, amplifying target nucleic acid in the micronuclei, wherein the target nucleic acid is associated with a cell proliferative disorder, and isolating the amplified target nucleic acid.

The term "isolated" as used herein refers to polynucleotides substantially free of other nucleic acids, proteins, lipids, carbohydrates or other materials with which they are naturally associated. Polynucleotide sequences of the invention include DNA, cDNA and RNA sequences which encode amplified extrachromosomal target gene or loci.

The method of the invention includes subjecting a cell suspected of having a cell proliferative disorder to conditions sufficient to produce micronuclei. Micronuclei refer to structures which preferably entrap extrachromosomal nucleic acid molecules and only infrequently contain chromosomes. In the method of the invention, a cell is subjected to an agent which induces micronuclei formation. Such agents include, but are not limited to inhibitors of DNA replication, DNA damaging agents, inhibitors of topoisomerase II, and membrane disrupting agents. Examples of such agents which induce micronuclei

formation include hydroxyurea, retinoic acid, dimethyl sulfoxide, guanazole, etoposide, proflavine, and difluoromethylornithine. Other agents having the function of those described herein will be known to those of skill in the art.

Hydroxyurea is utilized in the method of the invention at a concentration of about 1μM to 200μM, preferably from about 50μM to 150μM and most preferably from about 75μM to 100μM. Methods for evaluating the effectiveness of agents for inducing micronuclei include preparation of metaphase chromosomes and interphase nuclei and fluorescent *in situ* hybridization (FISH), as described in the illustrative EXAMPLES herein.

Isolation of the micronuclei from the cell is accomplished by physical separation, density gradient separation and/or immunoseparation. Such methods will be known to those of skill in the art. Preferably, the physical separation is differential centrifugation. Density gradient separation may be utilizing a medium such as Ficoll® or Percoll® (registered trademarks of Pharmacia), sucrose, or bovine serum albumin. Other comparable density gradient separation medium will be known to those of skill in the art or will be readily ascertainable. (See for example, *Current Protocols in Molecular Biology*, Ausubel, ed., Wiley & Sons, 1994; Coligan, *et al.*, *Current Protocols in Immunology,* Wiley Interscience, 1994).

Immunoseparation is optional and is performed by means of an antibody having the specificity of an anti-nuclear pore antibody or an anti-lamin antibody. Other antibodies having the specificity of an anti-nuclear pore antibody or an anti-lamin antibody and which bind to micronuclei are also useful in the immunoseparation process.

Following isolation of micronuclei from a cell, amplification is accomplished by polymerase chain reaction or other comparable means of amplification typically utilized by those of skill in the art. Oligonucleotide primers are used according to the invention and are employed in any amplification process that produces increased quantities of target nucleic acid or target nucleic acid sequence. Typically, one primer is complementary to

the negative (-) strand of the nucleotide sequence and the other is complementary to the positive (+) strand. Annealing the primers to denatured nucleic acid followed by extension with an enzyme, such as the large fragment of DNA Polymerase I (Klenow) or Taq DNA polymerase and nucleotides or ligases, results in newly synthesized + and - strands containing the target nucleic acid. Because these newly synthesized nucleic acids are also templates, repeated cycles of denaturing, primer annealing, and extension results in exponential production of the region (*i.e.*, the target nucleotide sequence) defined by the primer which is a discrete nucleic acid duplex with termini corresponding to the ends of the specific primers employed. Those of skill in the art will know of other amplification methodologies which can also be utilized to increase the copy number of target nucleic acid.

Preferably, the method of amplifying is by PCR, as described herein and as is commonly used by those of ordinary skill in the art. Alternative methods of amplification have been described and can also be employed as long as the target nucleic acid amplified by PCR using suitable primers is similarly amplified by the alternative means. Such alternative amplification systems include self-sustained sequence replication, 3SR, which begins with a short sequence of RNA of interest and a T7 promoter. Reverse transcriptase copies the RNA into cDNA and degrades the RNA, followed by reverse transcriptase polymerizing a second strand of DNA. Another nucleic acid amplification technique is nucleic acid sequence-based amplification (NASBA) which uses reverse transcription and T7 RNA polymerase and incorporates two primers to target its cycling scheme. NASBA can begin and end with either DNA or RNA and finish with either, and amplifies up to 10^8 copies within 60 to 90 minutes. Alternatively, nucleic acid can be amplified by ligation activated transcription (LAT). LAT works from a single-stranded template with a single primer that is partially single-stranded and partially double-stranded. Amplification is initiated by ligating a cDNA to a promoter oligonucleotide and within a few hours, amplification is 10^8 to 10^9-fold. The Qβ replicase system can be utilized by attaching an RNA sequence called MDV-1 to RNA complementary to a DNA sequence of interest. Upon mixing with a sample, the hybrid RNA finds its complement among the specimen's

mRNAs and binds, activating the replicase to copy the tag-along sequence of interest. Another nucleic acid amplification technique, ligase chain reaction (LCR), works by using two differently labeled halves of a sequence of interest which are covalently bonded by ligase in the presence of the contiguous sequence in a sample, forming a new target. The repair chain reaction (RCR) nucleic acid amplification technique uses two complementary and target-specific oligonucleotides, thermostable polymerase and ligase, and DNA nucleotides to geometrically amplify targeted sequences. A 2-base gap separates the oligonucleotide probes, and the RCR fills and joins the gap, mimicking normal DNA repair. Nucleic acid amplification by strand displacement activation (SDA) utilizes a short primer containing a recognition site for *Hinc*II with a short overhang on the 5' end which binds to target DNA. A DNA polymerase fills in the part of the primer opposite the overhang with sulfur-containing adenine analogs. Following amplification, *Hinc*II is added to cut the unmodified DNA strand. A DNA polymerase that lacks 5' exonuclease activity enters at the site of the nick and begins to polymerize, displacing the initial primer strand downstream and building a new one which serves as more primer. SDA produces greater than 10^7-fold amplification in 2 hours at 37°C. Unlike PCR and LCR, SDA does not require instrumented temperature cycling. Although PCR is the preferred method of amplification of the invention, these other methods can also be used to amplify the Amplified target nucleic acid locus as described in the method of the invention.

Primers which can be used for amplification of the target DNA sequence in the method of the invention embrace oligonucleotides of sufficient length and appropriate sequence so as to provide specific initiation of polymerization of a significant number of nucleic acid molecules containing the target nucleic acid. In this manner, it is possible to selectively amplify the specific target nucleic acid sequence containing the nucleic acid of interest. Specifically, the term "primer" as used herein refers to a sequence comprising a suitable number of deoxyribonucleotides or ribonucleotides, preferably at least eight, which sequence is capable of initiating synthesis of a primer extension product, which is

substantially complementary to a target nucleic acid strand. The oligonucleotide primer typically contains 15-22 or more nucleotides, although it may contain fewer nucleotides.

Experimental conditions conducive to amplification include the presence of nucleoside triphosphates and an agent for polymerization, such as DNA polymerase, and a suitable temperature and pH. The primer is preferably single stranded for maximum efficiency in amplification, but may be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent for polymerization. The exact length of primer will depend on many factors, including temperature, buffer, and nucleotide composition.

Primers are designed to be "substantially" complementary to each strand of the nucleotide sequence to be amplified. Substantially complementary means that the primers must be sufficiently complementary to hybridize with their respective strands under conditions which allow the agent for polymerization to function. In other words, the primers should have sufficient complementarity with the flanking sequences to hybridize therewith and permit amplification of the nucleotide sequence. Preferably, the terminus of the primer that is extended has perfectly base paired complementarity with the complementary flanking strand.

Oligonucleotide primers for use in the invention may be prepared using any suitable method, such as conventional phosphotriester and phosphodiester methods or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage, *et al.* (*Tetrahedron Letters*, 22:1859-1862, 1981). One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Patent No. 4,458,066.

Micronuclei nucleic acid amplified in accordance with the invention contains a specific known nucleic acid sequence or an unknown target nucleic acid. Thus, the nucleic acid starting materials that can be employed include, for example, DNA or RNA, including messenger RNA, wherein DNA or RNA may be single stranded or double stranded. If RNA is to be used as a template, enzymes, and/or conditions optimal for reverse transcribing the template to DNA are utilized. In addition, a DNA-RNA hybrid which contains one strand of each may also be utilized. Moreover, a mixture of nucleic acids may also be employed, or nucleic acids produced in a previous amplification reaction using the same or different primers may be utilized. The nucleic acid sequence to be amplified, may be a portion of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor fraction of a complex mixture, such as contained in whole human DNA.

The agent for polymerization in the amplification reaction may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Taq polymerase, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, polymerase muteins, reverse transcriptase, ligase, and other enzymes, including heat-stable enzymes (*i.e.*, those enzymes which perform primer extension after being subjected to temperatures sufficiently elevated to cause denaturation). Suitable enzymes will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each template strand of target nucleic acid. Generally, the amplification will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths. There may be agents for amplification, however, which initiate synthesis at the 5' end and proceed in the other direction, using the same process as described above. In any event, the method of the invention is not limited to the embodiments of amplification which are described herein.

The amplified product may be detected by analyzing it by Southern blots without using radioactive probes. In such a process, for example, a small sample of nucleic acid containing a very low level of target nucleotide sequence is amplified, and analyzed via a Southern blotting technique. The use of non-radioactive probes or labels is facilitated by the high level of the amplified signal. For determination of the identity of the isolated amplified target nucleic acid, probes which hybridize to known amplified sequences may be used first to positively identify the target sequence or to eliminate the possibility that the amplified sequence is a previously identified gene (*e.g.*, myc, neu, PRAD, MDR1).

Nucleic acids having an amplified target sequence detected in the method of the invention can be further evaluated, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the analysis of a specific DNA sequence such as PCR, oligomer restriction (Saiki, *et al.*, *Bio/Technology*, 3:1008-1012, 1985), allele-specific oligonucleotide (ASO) probe analysis (Conner, *et al.*, *Proc. Natl. Acad. Sci. USA*, 80:278, 1983), oligonucleotide ligation assays (OLAs) (Landegren, *et al.*, *Science*, 241:1077, 1988), fluorescent in situ hybridization (FISH) and the like. Molecular techniques for DNA analysis have been reviewed (Landegren, *et al.*, *Science*, 242:229-237, 1988).

DNA sequences amplified by any one of a variety of means, can be cloned by several methods. For example, the DNA can be isolated using hybridization procedures which are well known in the art. These include, but are not limited to : 1) hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences; 2) antibody screening of expression libraries to detect shared structural features and 3) synthesis by the polymerase chain reaction (PCR).

Hybridization procedures are useful for the screening of recombinant clones by using labeled mixed synthetic oligonucleotide probes where each probe is potentially the complete complement of a specific nucleic acid sequence in the hybridization sample which includes a heterogeneous mixture of denatured double-stranded nucleic acid. For

such screening, hybridization is preferably performed on either single-stranded nucleic acid or denatured double-stranded nucleic acid. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al., Nucleic Acid Research*, 9:879, 1981).

The analysis of specific DNA sequences encoding amplified target nucleic acid sequences can also be obtained by: 1) isolation of double-stranded DNA sequences from the genomic DNA; 2) chemical synthesis of a DNA sequence to provide the necessary codons for the polypeptide of interest; and 3) *in vitro* synthesis of a double-stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double-stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA. Of these three methods for developing specific DNA sequences for use in recombinant procedures, the isolation of genomic DNA isolates is the least common. This is especially true when it is desirable to obtain the microbial expression of mammalian polypeptides due to the presence of introns.

Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid- or phage-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay *et al., Nucl. Acid Res.* 11:2325, 1983). One method of isolating cDNA

sequences representative of the amplified target nucleic acid in the micronuclei also includes a subtractive library approach for isolating cDNA clones derived from mRNAs exhibiting higher or lower abundance.

A cDNA expression library, such as lambda gt11, can be screened indirectly for the expression product of the amplified target nucleic acid by identifying a polypeptide having at least one epitope, using antibodies specific for Amplified target nucleic acid. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of amplified target nucleic acid cDNA.

An amino acid sequence of a polypeptide can be deduced from a target nucleic acid utilizing the genetic code, however, the degeneracy of the code must be taken into account. Polynucleotides of the invention include sequences which are degenerate as a result of the genetic code. The polynucleotides of the invention include sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, as long as the amino acid sequence of an amplified target nucleic acid results in a functional polypeptide (at least, in the case of the sense polynucleotide strand), all degenerate nucleotide sequences are included in the invention.

There are other methods of amplification and cloning of the amplified target nucleic acid of the invention including microdissection-PCR approach which can generate PCR products for use as probes to identify phage, cosmid or YAC clones in existing libraries of normal human DNA. RNA arbitrarily primed PCR, "RAP" or "differential display" can be used to produce a DNA fingerprint of the transcribed RNA that is suitable for molecular cloning (Welsh, *et al, Nucl. Acids Res.*, 20:4965, 1992; Liang, *et al., Science*, 257:967, 1992; Wong, *et al., Int. J. Oncol.*, 3:3, 1993, all incorporated by reference, herein).

The amplified target nucleic acid of the invention is a gene which encodes a protein such as a transcriptional regulator, a growth factor receptor, an inhibitor of the cell cycle, a G-protein, and a cell cycle-associated gene. Examples of such genes include, but are not limited to *c-myc*, *N-myc*, *Her-2/neu*, *PRAD1*, *erbB-2*, and *H-ras*. The amplified target nucleic acid may also be a gene which is referred to as a drug-resistance gene. Amplification of such genes, including dihydrofolate reductase (DHFR), carbamyl-phosphate synthetase-aspartate transcarbamylase-dihydroorotase (CAD) and multidrug resistance gene-1 (MDR1), provide a mechanism for a cell to avoid cell death upon treatment with the appropriate drug.

The method of the invention allows isolation of an amplified target nucleic acid which may be a previously known or an unknown gene. One of skill in the art will be able to use an amplification method, such as PCR, to isolate the gene for further identification. Probes which identify known genes are known and available to those of skill in the art. By elimination, one can readily determine if an amplified nucleic acid is a previously identified gene or not.

A cell proliferative disorder may be for example, associated with increased transcription and translation of an amplified target DNA sequence. The term "cell-proliferative disorder" denotes malignant as well as non-malignant cell populations which morphologically often appear to differ from the surrounding tissue. For example, the method of the invention may be useful in diagnosing malignancies of the various organ systems, such as, for example, lung, breast, lymphoid, hematopoietic, gastrointestinal, and genito-urinary tract as well as adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer, non-small cell carcinoma of the lung, ovarian cancer, brain cancer, uterine cancer, bladder cancer, cancer of the small intestine, and cancer of the esophagus.

The method is also useful in diagnosing non-malignant or immunological-related cell-proliferative diseases such as psoriasis, pemphigus vulgaris, Behcet's syndrome, acute

respiratory distress syndrome (ARDS), ischemic heart disease, post-dialysis syndrome, leukemia, rheumatoid arthritis, acquired immune deficiency syndrome, vasculitis, lipid histiocytosis, septic shock and inflammation in general. Essentially, any disorder which is etiologically linked to amplified nucleic acid target sequence would be considered a cell proliferative disorder as described herein.

The materials for use in the method of the invention are ideally suited for a kit. Such a kit comprises an agent which induces micronuclei formation, and means for amplifying target nucleic acid in the micronuclei, wherein the means comprises the necessary enzyme(s) and oligonucleotides for amplifying the target nucleic acid from a cell suspected of having a proliferative disorder.

The following examples are intended to illustrate but not limit the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may alternatively be used.

EXAMPLES

Various cell lines that are known in the art to contain amplified nucleic acid sequences on extrachromosomal DMs, as well as cells suspected of having amplified nucleic acid sequences can be utilized in the method of the invention. The following illustrative examples utilized cell lines which are known to contain DMs having amplified oncogene sequences.

EXAMPLE 1

ANALYSIS OF CELL LINES WITH DMs

Cell Lines

An early passage (passage 46) of the HL60 promyelocytic leukemia cell line was obtained from S. Colins (Fred Hutchinson Cancer Center). This cell line contains 16-32 copies of the *MYC* oncogene, the majority of which localize to extrachromosomal molecules ranging from 250-kbp episomes to DMs (Collins, *et al., Nature,* 270:347-349, 1977; and Von Hoff, *et al., Proc. Natl. Acad. Sci., USA,* 85:4804-4808, 1988). Passage 67 subclone 173 contains a median of 8 DMs per cell and was used for this study (Van Hoff, *et al., supra*; and Von Hoff, *et al., J. Clin. Invest.,* 85:1887-1895, 1990). A previously described subclone of COLO 320DM (American Type Culture Collection; Quinn, *et al., Cancer Res.,* 39:4914-4924, 1979), which contains a median of 30 DMs per cell and an amplicon of 120-160 kbp (Von Hoff, *et al., supra*), was used. The COLO 320 HSR (HSR, homogeneously staining region) line has approximately the same number of copies of *MYC* dispersed at several chromosomal sites (Von Hoff, *et al., supra*). The NB4 neuroblastoma cell line (passage 20; kindly provided by J. Casper and V. Piaskowski, Milwaukee Children Hospital) has an ≈50-fold amplification of the *NMYC* gene localizing to ≈1000-kbp episomes and DMs (VanDevanter, *et al., J. Natl. Cancer Inst.,* 82:1815-1821, 1990). The SF188 glioblastoma multiform cell line, passage 220, has a 25-fold amplification of the *MYC* gene localizing to heterogeneously sized episomes, with a minimum size of 100 kbp, and DMs (Trent, *et al., Proc. Natl. Acad. Sci, USA,* 83:470-473, 1986).

The HL60 and COLO 320 cell lines were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), and NB4 and SF188 in RPMI 1640 medium containing 20% FBS and 2 mM glutamine. Based on previous work by Snapka and Varshavsky, *Proc. Natl. Acad. Sci., USA,* 807533-7537, 1983, HU (Squibb) was added on day 0 of culture at the concentrations indicated and was replaced each time the cells were passaged. All cells were passaged by a 1:10 dilution of confluent cultures every 3-7 days. Cell growth was determined with a hemocytometer.

Evaluation of Cells for Micronuclei and Localization of *MYC* Genes

Micronuclei were scored in preparations of metaphase chromosome spreads and interphase nuclei (Von Hoff, *et al., supra*; and Naylor, *et al., Methods Enzymol.,* 151:279-292, 1987). The cells were exposed to Colcemid (0.1 μg/ml; GIBCO) for 1-3 hours, incubated in 0.075 M KCl for 20 minutes, fixed in methanol/glacial acetic acid (3:1), and dropped on wet slides. Portions of tumors that had been established *in vivo* were either used immediately for preparation of metaphase spreads or reestablished in cell culture to enable a comparison of the number of *MYC* DMs per cell under various growth conditions.

The *MYC* cosmid (Yuxin Yin, Salk Institute) and centromere probes (Oncor, Gaithersburg, MD) used for *in situ* hybridization were labeled with biotin-16-dUTP (Boehringer Mannheim) by nick-translation with a reaction mixture containing all four dNTPs (Pharmacia). Fluorescent *in situ* hybridization (FISH) was conducted as described by Pinkel, *et al., (Proc. Natl. Acad. Sci., USA,* 83:2934-2938, 1986).

Exposure of Cells to Hydroxyurea

All cells were continuously exposed to concentrations of hydroxyurea (Sigma) of 50, 100, and 200 μM.

EXAMPLE 2

PURIFICATION OF MICRONUCLEI FROM CELLS CONTAINING AMPLIFIED GENES AS DM AND/OR EPISOMES

Cell lines

Human Colo320DM or Colo320HSR neuroendocrine tumor cells (Alitalo, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 80, 1707-1711 (1983)) were provided by D.D. Vonhoff (University of Texas, San Antonio) and grown in RPMI1640 medium supplemented with 10% fetal calf serum. When the cell density reached $1.5x10^6$ cells/ml, a subculture was made by diluting to $2.5x10^5$ cells/ml with growth medium. The locations of amplified c-*myc* genes to DMs or HSRs were confirmed by FISH using c-*myc* cosmid DNA (from Yuxin Yin, Salk Institute). B-1/50 cell line (Yeung, *et al.*, *J. Biol Chem.*, 258, 8338-8345 (1983); Nonet, *et al.*, *Genomics* 15, 543-558 (1993)) was provided by R. Kellems (Texas Medical Center) and grown in DMEM supplemented with 1x MEM nonessential amino acids (GIBCO BRL), 15% heat-activated horse serum, 50 μM 2'-deoxycofomycin, 1.1 mM adenosine, 20 μM azaserine, and 1 mM uridine. 2'-Deoxycofomycin was obtained from the National Cancer Institute. XEW8.2.3 cell line (Carine, *et al.*, *Somat. Cell Mol. Genet.*, 12, 479-491 (1986; Carine, *etal.*, *Somat. Cell Mol. Genet.* 15, 445-460 (1989)) was developed by and provided from I. Scheffler (University of California, San Diego) and maintained in DMEM supplemented with 10% fetal calf serum. Human diploid WS-1 cell line was obtained from American Type Culture Collection (CRL 1502) (ATCC, Rockville, MD) and maintained in DMEM supplemented with 10% heat activated fetal calf serum and 1 x MEM nonessential amino acids. All cells were grown at 37 °C with 7% CO_2. Hydroxyurea (Sigma) of indicated final concentration was added to the culture when it was subcultured to lower cell density. Cells were grown in the presence of hydroxyurea for, unless otherwise noted, 3 days.

Purification of micronuclei

This procedure was developed, in some portion, based on the protocol for the isolation of intact nuclei (Janssen, K. *et al., Current Protocols in Molecular Biology*, p4.10.1-4.10.11, John Wiley & Sons, Inc., NY). FIGURE 1 shows a flow chart of the isolation protocol used for isolation of micronuclei and amplified nucleic acid sequences contained within the micronuclei. The cells (~ $1x10^9$ cells) were treated with 100 μM hydroxyurea for 3 days, harvested and washed twice with DMEM without serum by centrifuging 200 x g for 5 min. The cell precipitate was resuspended in 20 ml of prewarmed DMEM containing cytochalasin B (10 μg/ml) and incubated for 30 min at 37 °C. After collection at 200 x g for 5 min, cells were resuspended in 10 ml of prechilled lysis buffer (10 mM Tris-Cl, 2 mM Mg-acetate, 3 mM $CaCl_2$, 0.32 M sucrose, 0.1 mM EDTA, 1 mM Dithiothreitol, 0.5% (v/v) Nonidet P-40, 0.15 mM spermine, 0.75 mM spermidine and 10 μg/ml cytochalasin B, pH 8.5), and Dounce homogenized (5 to 10 strokes using loose fitting pestle). The release of micronuclei from cytoplasm or nuclei was confirmed by mixing small portion of homogenate with equal volume of PBS containing 2 μg/ml of DAPI and 0.1 μg/ml of PI, and examining under fluorescence microscope using triple band path filter. Then, the homogenate was combined with the equal volume of 2M sucrose buffer (10 mM Tris-Cl, 2M sucrose , 5 mM Mg-acetate, 0.1 mM EDTA, 1mM dithiothreitol, pH 8.0, 4 °C), and was layered onto the top of sucrose stepwise gradient (10, 5, and 5 ml of sucrose buffer containing 2 M, 1.8 M, and 1.6 M of sucrose respectively from the bottom of tube), and centrifuged in SW28 rotor, 14,000 rpm, 45 min at 4 °C. After centrifugation, the pellet and the interphase between 2 and 1.8M sucrose layer, which contain nuclei and micronuclei, were recovered, and washed twice by PBS- by centrifuging 1,000 x g, 20 min at 4 °C. The suspension (3ml) was then layered on the top of linear BSA gradient (0.5 to 4% in PBS, total volume 40 ml, made in 50 ml disposable syringe) (Dhar, *et al., Somat. Cell Mol. Genet.*, 10:547-559, 1984), and allowed to sit at 4 °C for 4 hours. Fractions (2ml each) were taken from the top of gradient, diluted by PBS and centrifuged at 1,000 x g for 20 min. The precipitate from fractions 1 to 8 were suspended in PBS, mixed and re-fractioned by the BSA gradient sedimentation as above. Small portion of each fraction was stained with DAPI and

examined under fluorescence microscope. About 90% of DAPI positive particles present in fractions 1 to 4 were shown to typical micronuclei based on the size, the shape and the property of DAPI staining. Fractions 1 to 4 were pooled and treated with DNase I(5 μg/ml) and RNase A (40 μg/ml) for 30 min at 37 °C. This fraction was determined to be purified micronuclei thereafter. A portion of purified micronuclei was fixed by methanol/ acetic acid (3/1), and examined by *in situ* hybrization using c-*myc* cosmid probe. Remaining samples were treated with proteinase K (60 μg/ml) and 0.05% triton X-100 for 60 min at 50% followed by the inactivation of the enzyme at 94 °C for 12 min, and used for the gene quantitation by PCR or the generation of FISH probe.

Gene quantitation by PCR

The amount of c-*myc* gene amplified on DMs in Colo320DM cells was quantitated and control β-globin gene, single copy on chromosome 11, by competitive PCR procedure as described (Siebert, *et al.*, *BioTechniques*, 14, 244-249, 1993; Forster, *et al.*, *BioTechniques*, 16, 1006-1008, 1994). The sequences of primers used for c-*myc* gene were myc-C, 5'd(CTG GGA TCT TCT CAG CCT AT)3' (SEQ ID NO:1) and myc-D, 5'd(ACT CCT CTC ACC ATG AAG GT)3' (SEQ ID NO:2). The sequences of primers used for β-globin were IVS-I, 5'd (GTA TCA TGC CTC TTT GCA CC)3' (SEQ ID NO:3), and IVS-L, 5'd(AAG GGC CTA GCT TGG ACT CA) (SEQ ID NO:4). The primer set of myc-C and myc-D amplifies 400 bp product from human c-*myc* gene intron 2, and the primer set of IVS-I and IVS-L amplifies 214 bp product form human β-globin gene intron 2. Internal standards for each genes were prepared by PCR amplification using c-*myc* or β-globin primer pairs from salmon DNA or *Saccharomyces pombe* DNA, respectively. At that time, annealing temperature was lowered to 42 C or 47°C for c-*myc* and β-globin, respectively. The products were separated by agarose gel electrophoresis, and the bands of ca. 200bp or ca. 400 bp for c-*myc* or β-globin, respectively, were excised. Standard DNAs in the excised bands were further purified by successive 3 rounds of PCR amplification at higher annealing temperature (63 °C) and fractionation by agarose gel electrophoresis. The amount of final PCR products, each which gave single band in agarose gel electrophoresis, were quantitated by the intensities of the

ethidium bromide-stained bands in agarose gel, and used as the standard DNA for the quantitation of test DNA.

For this purpose, a series of PCR reactions were made containing equal amount of test DNA and serial 2-fold diluted standard DNA. Each tube (10μl) contain 1 x Taq buffer (Invitrogen; N for c-*myc*, J for β-globin), 0.2 mM each of dNTP, 20 ng each of primers, 0.2 μl of test DNA, serially diluted known amount of standard DNA, and 0.4 u of Taq polymerase (Boehringer Manheim). The tubes were heated to 95 °C for 3 min followed by 40 cycles at 94 °C for 1 min, 63 °C for 1 min, 72 °C for 2 min. After the end of PCR, the products were seperated by agarose gel electphoresis, stained with ethidium bromide and the intensities of the products from test and standard DNA, differing in the size, were compared and evaluated.

The generation of FISH probe from purified micronuclei

DNA in the proteinase K-treated micronuclei preparation was uniformly amplified by randomly primed PCR as reported by Telenius, *et al.* (Telenius, *et al., Genes Chrom. Cancer*, 4, 257-263, 1992) Briefly, an initial 8 cycles of PCR (94 °C for 1 min, 30 °C for 4 min, 37 °C for 2.5 min) was conducted in 5 μl of 1 x Sequenase reaction buffer (USB), 0.2 mM each dNTP, and 10 μM of Telenius primer by adding 0.2 u of Sequenase (Ver. 2.0 USB) at each cycle. After this step, a conventional PCR was performed in the same tube by adding 50 μl of reaction mixture containing 1 x Taq buffer B (Invitrogen), 0.2 mM each of dNTP, 2 μM of Telenius primer, 2.5 u of Taq DNA polymerase (Boehringer Mannheim). The reaction was heated to 95 °C for 3 min followed by 35 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min. Amplified products were FITC-labeled by "Prime-it Fluor Fluorescence labeling kit" (Stratagene) primed with Telenius primer instead with random hexanucleotides supplied in the kit. In some experiments, uniformly amplified products were labeled by biotin using "BioPrime DNA System" (Life Technologies) as per the manufacturer's protocol.

FISH

Metaphase spreads were prepared as described and treated with RNase (100 μg/ml in 2 x SSC, 37 °C, 60 min.) Hybridization of the FISH probes was as described (Pinkel, *et al.*, *Proc. Natl. Acad. Sci., USA*, 83:2934-2938, 1986). Briefly, for each hybridization, 50 ~100 ng of probe was used in 15 μl hybridization mixture (containing 50% formamide, 10% dextran sulfate, 2x SSC, 6 μg salmon sperm DNA, and 3 μg human or mouse COT I DNA (BRL) depending on the probe species used) which was denatured at 75 °C for 5 min followed by 42 °C, 30 min. The slides with metaphase spreads was denatured in 70% formamide, 2x SSC at 70~72 °C for 2 min, rinsed in ice cold 70%, 85% and 100% ethanol for 3 min each, and air dried. The hybridization with probes was done at 37 °C in a moist chamber overnight. The slide was then washed three times in 50% formamide, 2x SSC at 45 °C for 3 min each, three times in 2xSSC at 45°C for 3 min each, and one time in 0.1 x SSC at 60 °C for 10 min. The slide was viewed at this point when FITC labeled probes were used. Alternatively, the hybridization signal of biotin labeled probe was detected with one layer of FITC-conjugated avidin (Vector) and amplified with biotinylated anti-avidin (Vector) and a second layer of FITC-conjugated avidin. The slide was counter stained with 0.5 μg/ml of propidium iodide in Vectashield (Vector) and was examined with a Zeiss fluorescence microscope equipped with appropriate epifluorescence filters.

The results of the FISH studies confirmed the isolation of micronuclei and DMs by the method described herein. FIGURE 2a shows the frequency of micronuclei having c-myc versus the total number of interphase nuclei in COLO 320DM cells. FIGURE 2b shows the frequency of micronuclei in interphase nuclei in B-1/50 cells (mouse cells having amplification of adenosine deaminase gene). FIGURE 2c shows the frequency of micronuclei in interphase nuclei in XEW 8.2.3 cells (CHO cells having human centromere sequences and minichromosome). FIGURE 3 shows DNA in purified micronuclei from COLO 320DM cells (FITC labeled) and FISH hybridized to COLO 320DM metaphase spreads (x 1000 magnification).

FIGURE 4 shows micronuclei formed in COLO 320DM cells and FISH hybridized to c-myc cosmid DNA and detected by FITC (x1000 magnification). FIGURE 5 shows purified micronuclei from COLO 320DM cells treated with HU (100 uM) for 3 days and FISH hybridized to c-myc cosmid DNA (x400 magnification).

Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

CLAIMS

1. A method for isolating an amplified target nucleic acid associated with a cell proliferative disorder comprising:

subjecting a cell suspected of having a cell proliferative disorder to conditions sufficient to produce micronuclei;

isolating the micronuclei from the cell;

amplifying target nucleic acid in the micronuclei, wherein the target nucleic acid is associated with a cell proliferative disorder; and

isolating the amplified target nucleic acid.

2. A method for isolating an amplified target nucleic acid associated with a cell proliferative disorder comprising:

isolating the amplified target nucleic acid wherein said target nucleic acid is associated with a cell proliferative disorder and is located in micronuclei isolated from a cell(s) subjected to conditions sufficient to produce micronuclei.

3. A method for isolating an amplified target nucleic acid associated with a cell proliferative disorder comprising:

amplifying target nucleic acid in micronuclei isolated from cells subjected to conditions sufficient to produce micronuclei, wherein said target nucleic acid is associated with a cell proliferative disorder; and

isolating the amplified target nucleic acid.

4. A method for isolating an amplified target nucleic acid associated with a cell proliferative disorder comprising:

isolating micronuclei from a cell subjected to conditions sufficient to produce micronuclei;

amplifying target nucleic acid in the micronuclei wherein the target is associated with a cell proliferative disorder; and

isolating the amplified target nucleic acid.

5. The method of claim 1, wherein the cell is subjected to an agent which induces the formation of micronuclei in a cell.

6. The method of claim 5, wherein the agent is selected from the group consisting of an inhibitor of DNA replication, a DNA damaging agent, an inhibitor of topoisomerase II, and a membrane disrupting agent.

7. The method of claim 6, wherein the agent is hydroxyurea.

8. The method of claim 1, wherein isolation of micronuclei is accomplished by physical separation, density gradient separation and/or immunoseparation.

9. The method of claim 8, wherein physical separation is differential centrifugation.

10. The method of claim 8, wherein immunoseparation is by means of an antibody having the specificity of an anti-nuclear pore antibody.

11. The method of claim 1, wherein amplifying is accomplished by polymerase chain reaction.

12. The method of claim 1, wherein the target nucleic acid is a gene which encodes a protein selected from the group consisting of a transcriptional regulator, a growth factor receptor, an inhibitor of the cell cycle, a G-protein, and a cell cycle-associated gene.

13. The method of claim 12, wherein the target nucleic acid is a gene selected from the group consisting of c-myc, N-myc, Her-2/neu, PRAD1, erbB-2, and H-ras.

14. The method of claim 1, wherein the target nucleic acid is a drug-resistance gene.

15. The method of claim 14, wherein the drug resistance gene is selected from the group consisting of dihydrofolate reductase (DHFR), carbamylphosphate synthetase-aspartate transcarbamylase-dihydroorotase (CAD) and multidrug resistance gene-1 (MDR1).

16. The method of claim 1, wherein the cell is derived from a tissue selected from lung, breast, colon, ovary, blood, brain, bladder, and uterus.

17. A kit useful for the isolation of target nucleic acid associated with a cell proliferative disorder, the kit comprising an agent which induces micronuclei formation, and means for amplifying target nucleic acid in the micronuclei, said means comprising the necessary enzyme(s) and oligonucleotides for amplifying said target nucleic acid from a cell suspected of having a proliferative disorder.

18. The kit of claim 17, wherein the agent is selected from the group consisting of an inhibitor of DNA replication, a DNA damaging agent, an inhibitor of topoisomerase II, and a membrane disrupting agent.

19. The kit of claim 18, wherein the agent is hydroxyurea.

20. The kit of claim 17, further comprising a density gradient separation medium.

21. The kit of claim 17, further comprising an antibody having the specificity of an anti-nuclear pore antibody.

22. The kit of claim 17, wherein the target nucleic acid is a gene which encodes a protein selected from the group consisting of a transcriptional regulator, a growth factor receptor, an inhibitor of the cell cycle, a G-protein, and a cell cycle-associated gene.

23. The kit of claim 17, wherein the target nucleic acid is a gene selected from the group consisting of c-myc, N-myc, Her-2/neu, PRAD1, erbB-2, and H-ras.

24. The kit of claim 17, wherein the target nucleic acid is a drug-resistance gene.

25. The kit of claim 24, wherein the drug resistance gene is selected from the group consisting of dihydrofolate reductase (DHFR), carbamylphosphate synthetase-aspartate transcarbamylase-dihydroorotase (CAD) and multidrug resistance gene-1 (MDR1).

26. The kit of claim 17, wherein the cell is derived from a tissue selected from lung, breast, colon, ovary, blood, brain, bladder, and uterus.

ABSTRACT

The present invention provides a method for the isolation of extrachromosomal amplified nucleic acids that are associated with a cell proliferative disorder. Isolation and further identification of such genes is critical for diagnosis, prognosis, and course of therapy.

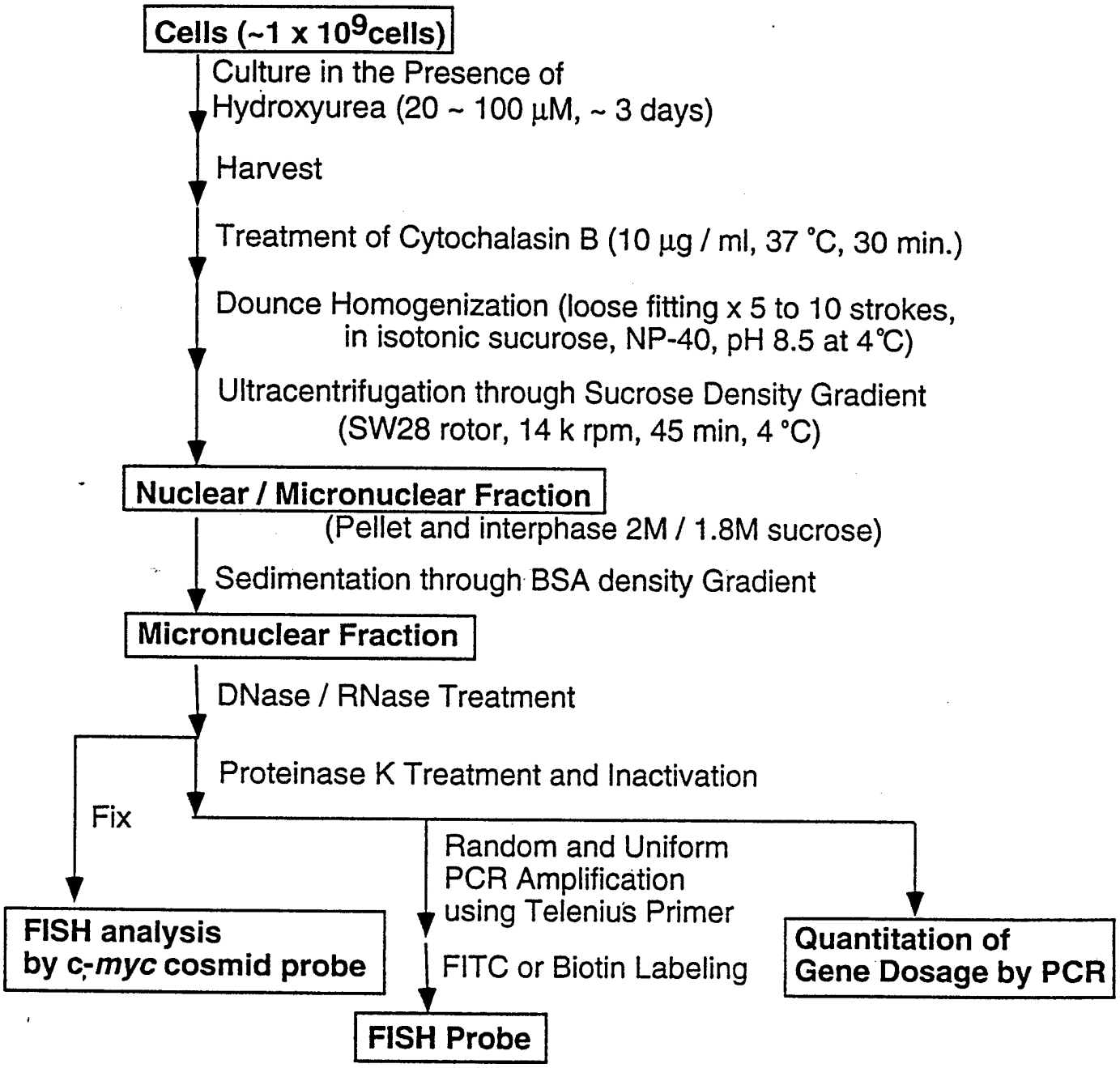

Cells (~1 x 10^9cells)
Culture in the Presence of Hydroxyurea (20 ~ 100 µM, ~ 3 days)
Harvest
Treatment of Cytochalasin B (10 µg / ml, 37 °C, 30 min.)
Dounce Homogenization (loose fitting x 5 to 10 strokes, in isotonic sucurose, NP-40, pH 8.5 at 4°C)
Ultracentrifugation through Sucrose Density Gradient (SW28 rotor, 14 k rpm, 45 min, 4 °C)
Nuclear / Micronuclear Fraction
(Pellet and interphase 2M / 1.8M sucrose)
Sedimentation through BSA density Gradient
Micronuclear Fraction
DNase / RNase Treatment
Proteinase K Treatment and Inactivation
Fix
Random and Uniform PCR Amplification using Telenius Primer
FITC or Biotin Labeling
FISH analysis by c-myc cosmid probe
FISH Probe
Quantitation of Gene Dosage by PCR

FIGURE 1

FIGURE 2a

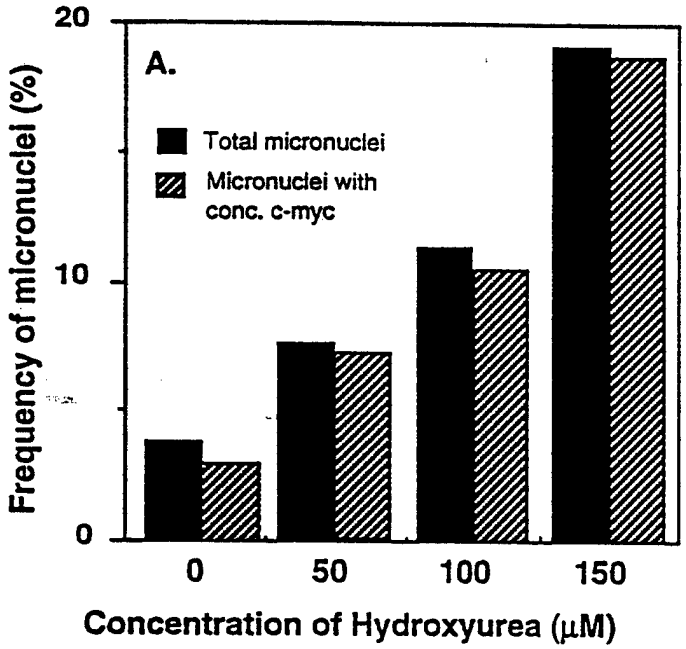

20
10
0
A.
Total micronuclei
Micronuclei with conc. c-myc
Frequency of micronuclei (%)
0
50
100
150
Concentration of Hydroxyurea (μM)

FIGURE 2b

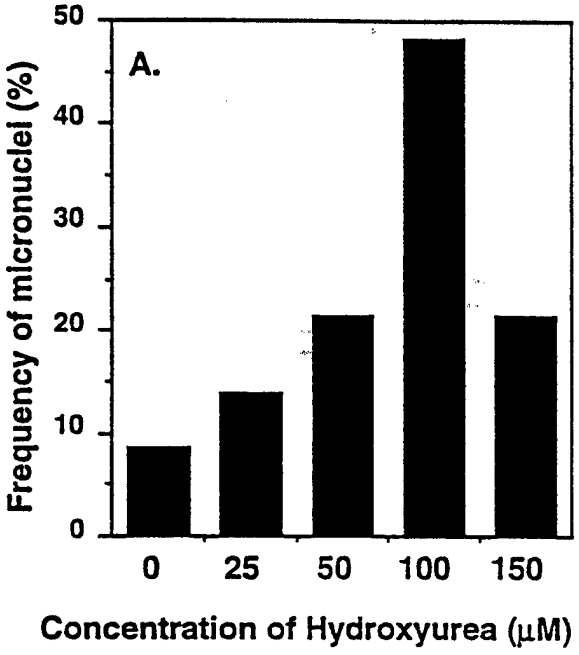

A.
Frequency of micronuclei (%)
50
40
30
20
10
0
0
25
50
100
150
Concentration of Hydroxyurea (μM)

FIGURE 2c

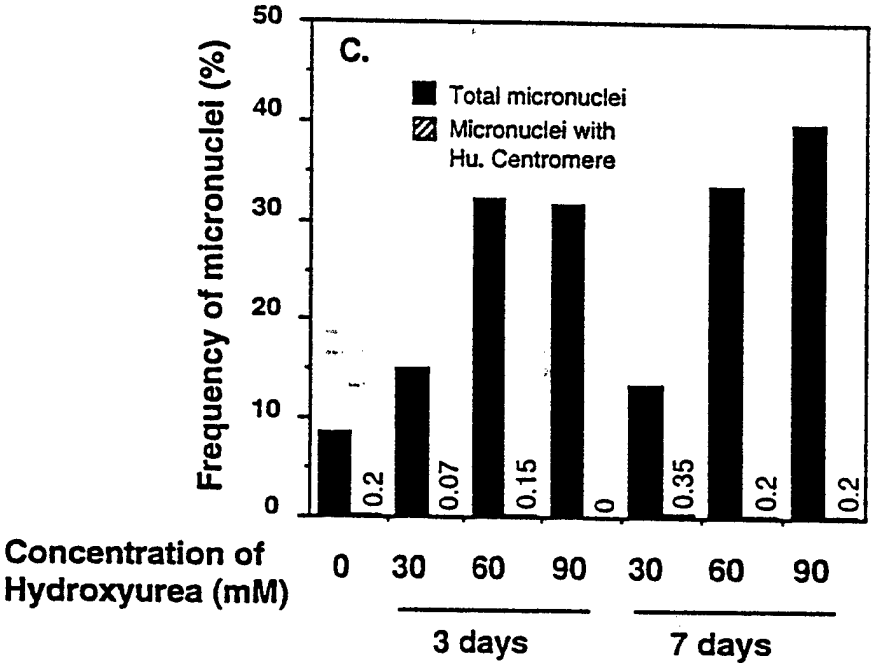

C.
Total micronuclei
Micronuclei with Hu. Centromere
Frequency of micronuclei (%)
50
40
30
20
10
0
0.2
0.07
0.15
0
0.35
0.2
0.2
Concentration of Hydroxyurea (mM)
0
30
60
90
30
60
90
3 days
7 days

FIGURE 3

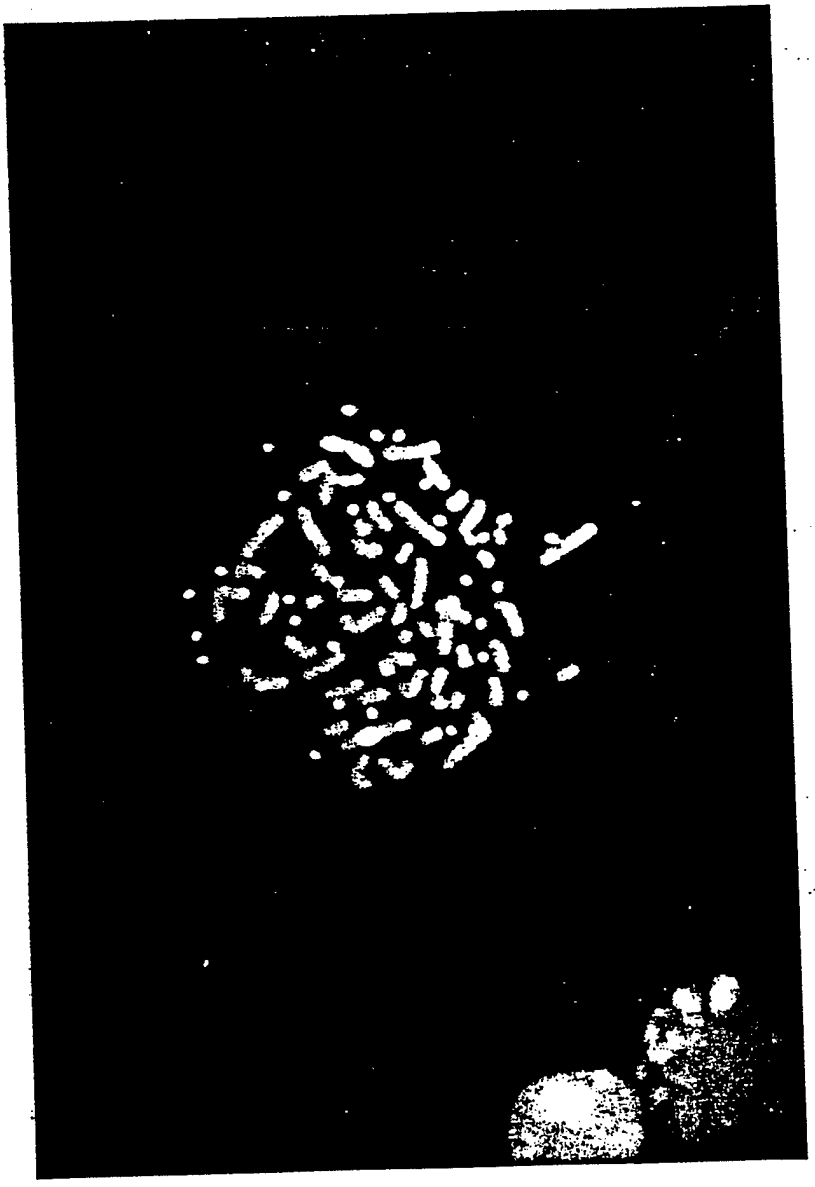


FIGURE 4

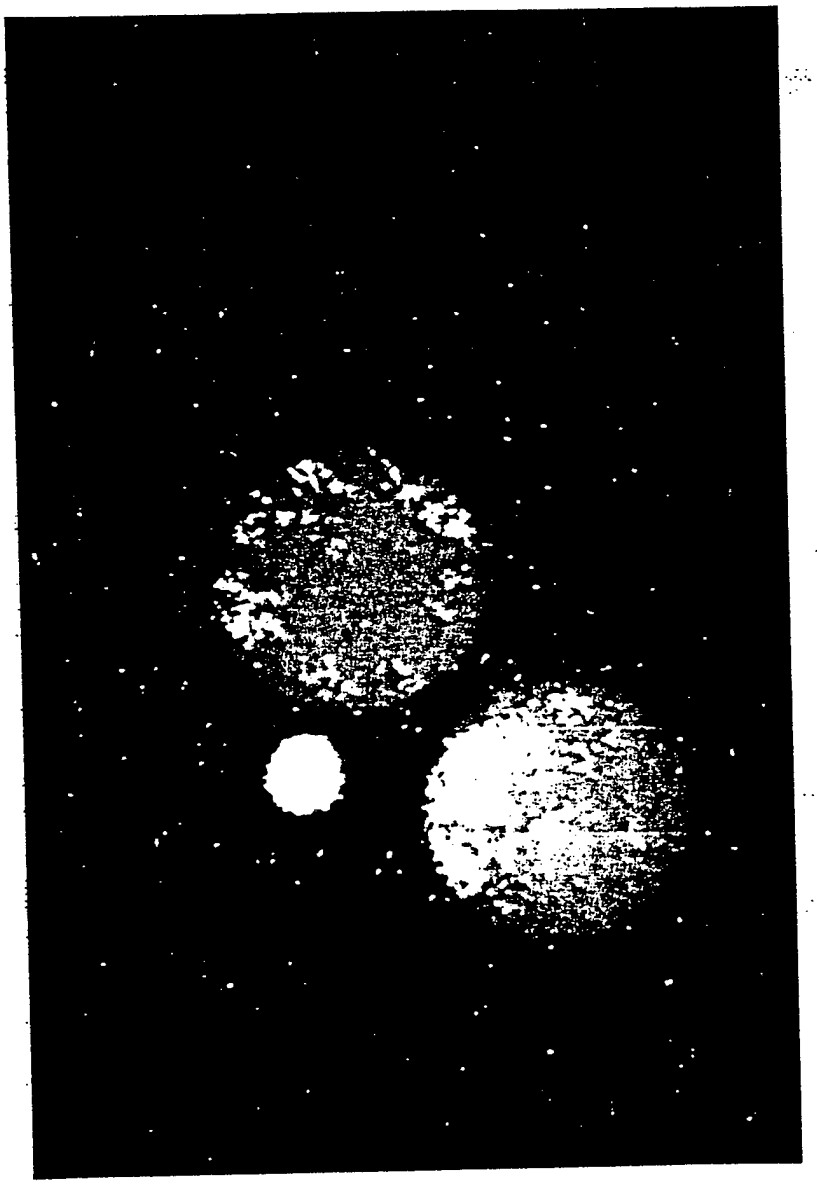


FIGURE 5